16 Changes of cellular redox homeostasis and protein folding in diabetes

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Chaperones are conserved and abundant proteins of the cell. They not only help to fold the newly synthesized proteins to get their final structure, but are also involved in many other events of the cellular life, such as signal transduction or protein degradation. A subset of chaperones takes part in the the formation of disulfide bonds, ion pairs and in the prolin cis-trans isomerisation of folding proteins. Disulfide bond formation is necessary for the active, functioning state of most secreted or plasma membrane proteins. Chaperones involved in disulfide bond formation need a highly regulated redox environment, which is provided by the lumen of the endoplasmic reticulum. The redox balance is maintained by many enzymatic systems, as well as by a permanent supply of coenzymes taking part in the electron transport chain. Diabetes is a well known metabolic disease, where one of the general symptoms is the presence of extensive oxidative stress. Here we give an overview of the role of oxidative homeostasis and chaperones in protein folding and summarize our current knowledge on changes of both redox balance and chaperone function in diabetes.

PROTEIN FOLDING

Protein folding is characterized by three major steps *in vitro* [1-5]. The first step is the formation of the secondary structure. In most of the cases folding starts with the formation of alpha-helices, and the formation of beta-sheets and beta-loops takes place afterward. In the end of this first step the unfolded protein is collapsed, and a more or less stable intermediate is formed, the *molten globule*. This step needs only a short time and usually goes without any support.

The partially folded state of molten globules can be characterized by a developed secondary structure but lacking a final tertiary structure [3–5]. Molten globules have large hydrophobic surfaces, therefore can easily aggregate. The volume of molten globules, however, is almost as small as that of the functional, folded protein.

The *last steps* of protein folding are slow, rate-limiting steps. During this process the inner, hydrophobic core of the protein gets organized, and unique, high-energy bonds are formed, such as disulfide bridges, ion-pairs, and the isomerization of proline cis/trans peptide bonds occurs [6, 7]. The free energy gain of these processes enables the formation of local, thermodynamically unstable, ìhigh-energy„ protein structures, which are maintained by the thermodynamically stable conformation of the rest of the protein. These ìhigh-energy„ segments of proteins can stabilize themselves by forming complexes with another molecule, thus they often serve as active centers of enzymes or as contact surfaces between various proteins involved *e.g.* in signal transduction.

THE ROLE OF MOLECULAR CHAPERONES

Protein folding is not a straightforward process. It can lead to dead-end streets, reverse reactions, futile cycles. Fully folded, native proteins always co-exist with various forms of molten globules and with traces of remaining unfolded specii. Aggregation of unfolded proteins and of molten globules is a great danger, which would drive the majority of folding intermediates to a nonproductive side-reaction, much before reaching their fully folded, competent state. Molecular chaperones serve to prevent this (Table 1). They recognize and cover hydrophobic surfaces successfully competing with the aggregation process [8, 9].

Table 1

Major classes of molecular chaperones

Most important eukaryotic representatives[a]	Reviews
small heat shock proteins (e.g. Hsp27[b])	26
Hsp60	8, 65
Hsp70 (Hsc70[c], Grp78)	8, 65
Hsp90	10, 11
Hsp104	66
Peptidyl-prolyl *cis/trans*-isomerases	7, 67
Protein disulfide isomerases (PDI-s)	6, 68

[a]Co-chaperones (chaperones which help the function of other chaperones listed) were not included in this table, albeit almost all of these proteins also possess a traditional„ chaperone activity in their own right. Several chaperones of the endoplasmic reticulum (e.g. calreticulin, calnexin, etc.), which do not belong to any of the major chaperone families, as well as some heat shock proteins (e.g. ubiquitin), which do not possess chaperone activity were also not mentioned. [b]The abbreviation Hsp„ and Grp„ refer to heat shock proteins, and glucose regulated proteins, chaperones induced by heat shock or glucose deprivation, respectively. Numbers refer to their molecular weight in kDa. [c]Hsc70 denotes the cognate (constitutively expressed) 70 kDa heatshock protein homologue in the cytoplasm.

Besides this aggregation-preventing role, the chaperones may aid protein folding in a direct way, by facilitating the transformation of unfolded specii to the fully folded protein. However, they may also unfold the incorrectly folded proteins, thus giving them another chance for correct folding. These two processes may go in parallel, or may be characteristic to different chaperone/target pairs [8, 9].

Besides directing the folding of newly sythesised proteins, chaperones have many other roles in the cellular events. Certain receptors, e.g. the glucocorticid receptors have large, hydrophobic binding site for the hormone. Hsp90, the most abundant chaperone of the cytoplasm prevents the binding site of these receptors from a collapse or aggregation [9–11].

ABBREVIATIONS: ER: endoplasmic reticulum; GSH, reduced glutathion; GSSG: oxidized glutathion; Hsp: heat shock protein; PDI: protein disulfide isomerase.

Chaperones are also involved in protein degradation, directing the selected, misfolded or covalently modified proteins toward the proteasome system [12]. Proteasomal degradation can occur only when the target protein is unfolded. So chaperones are also needed for this mechanism.

Some of the chaperones are inducible chaperones, which are sythesized during various forms of stress, when the rate of protein damage is higher, and there is an elevated demand of chaperones. In the case of stress, chaperones work in a rather passive way. Here chaperones just simply catch the damaged proteins, and protect them from aggregation. When the stress is over, and the normal cellular function is restored, chaperones release trapped proteins, and let them to be refolded or degraded [9].

THE ROLE OF REDOX HOMEOSTASIS IN PROTEIN FOLDING

On the contrary to the reduced environment of the cytoplasm, the average redox potential of the endoplasmic reticulum (ER) is about ñ160 mV, but theoretical calculations and some experimental results suggest that redox potential gradients and redox potential inhomogenities are typical of the subcompartment. The ER redox potential was thought to be mantained mostly by the glutathione/glutathione-disulfide redox buffer (GSH:GSSG=1 trough 3:1), and can be described by the thiol/disulfide ratio [13]. However, there are many other systems, which are able to influence ER thiol metabolism. The *in vitro* mechanisms to alter the redox state include a NADPH-dependent oxigenase and the vitamin-K redox cycle. There is an increasing number of evidence about the participation of different sulfhydryl-oxidases in the regulation of ER redox homeostasis of eukaryotic organisms [14]. In yeast models the direct role of hem, ubiquinone, Fe-S clusters and the exclusive role of molecular oxygen were recently refuted [15]. The possible involvement of flavin adenine dinucleotide (FAD) in the electron transport necessary to maintain ER redox homeostasis was also documented on yeast, where the addition of FAD accelerated the disulfide bridge formation by the ER-resident enzyme, ERO1p [15]. The yeast lumenal ER protein, ERV2 was also described as a flavoenzyme. ERV2 is able to accelerate O_2-dependent disulfide bridge formation under aerobic conditions [16].

Besides flavin adenine dinucleotide, an increasing number of evidence supports the involvement of an other, well known redox system in the regulation of the ER redox state. Ascorbate/dehydroascorbate concentration is in milimolar range in the ER lumen, and asorbate is a very important cofactor of the enzymes catalyzing prolyl- and lysyl-hydroxylation. Cytoplasmic ascorbic acid is first oxidized and dehydroascorbic acid is transported to the lumen by facilitated diffusion. Inside, an increased concentration of dehydroascorbic acid helps to mantain the transitional redox state, and takes part in the oxidation of GSH and protein thiols [17]. Moreover, protein disulfide isomerase itself has dehydroascorbate reductase activity [18]. The membrane-bound antioxidant agent, tocopherol was also mentioned as a possible contributor of the electron transport by ascorbic acid [19].

The importance of the glutathione/glutathione-disulfide redox buffer was recently assessed. The estimated redox potential of the ER (–160 mV) correlates with the current GSH/GSSG ratio, and its total concentration (1 to 2 mM) is high enough to affect redox environment and protein redox states [6]. However, the processes setting the balance between GSH and GSSG have not yet been clearly identified. Glutathione synthetase, an enzyme responsible for the *de novo* GSH generation, is located only in the cytoplasm, so GSH must enter to the ER lumen through transporters. A much faster GSSG transport was hypothesized to sustain the oxidative environment, but recent data are quite controversial on this topic [15]. Another possible way for the increase of GSSG concentration is described by some publications involving specific enzymatic GSSG generation by intraluminal redox enzymes [20].

The next chapter of glutathione research, its role on the protein folding process, is also under re-evaluation today. The increasing importance of GSH is underlined as a counterbalance for the ERO1p-mediated oxidation in yeast. According to this model, the oxidizing equivalents (whose precise nature is still unidentified) come from the cytosol, and are transmitted through the ER membrane by the ERO1p protein. Oxidation proceeds *via* the protein disulfide isomerases and finally reaches the secreted proteins. To avoid hyperoxidizing conditions a part of these proteins (and perhaps ERO1p itself) are reduced by GSH. This process is probably responsible for the

relatively low ER GSH/GSSG ratio [21]. The final step of ER glutathione metabolism is the secretion of GSH, which occurs by the vesicular transport system. The concentration of the glutathione in the vesicles targeting secreted and membrane surface proteins to the cell membrane is about 1 mM [22].

In all aerobic organisms active oxygen species are produced even under physiological conditions. A variety of antioxidant systems exists in the cytoplasm to diminish the oxidative damage. This important task is performed by enzymes like superoxide dismutase, peroxidase or catalase, or by antioxidants, such as ascorbic acid, coenzyme Q and glutathione (GSH), which can be easily oxidized, and provide a large redox buffer capacity for the cell. Gluthationylation, i.e. thiol-disulfide exchange between a cysteine residue in a protein and the oxidized form of glutathione (GSSG) occurs in oxidative stress. This process serves as a redox-dependent regulator of various protein functions [23], like the inactivation of the AP-1 or p53 transcription factors, the PTP-1B phosphotyrosine phosphatase [24] or the cAMP-dependent protein kinase [25].

There are numerous other systems, which specifically protect the sensitive molecules, mostly proteins from oxidative damage. In this process traditional chaperones (Table 1), e.g. the small heat shock proteins and Hsp70 can also serve as cytoplasmic "antioxidants" (Table 2). They protect their target proteins by covering their sensitive sites or by changing the overall redox status. Sometimes all these mechanisms are not effective enough, and the oxidative damage prevails. In this case Hsp-s capture denatured proteins and hold them until their (quite seldom) refolding or degradation.

These mechanisms are connected to each other in many ways. Small heat shock proteins elevate reduced glutathione levels by promoting an increase in glucose-6-phosphate dehydrogenase activity and by a somewhat smaller activation of glutathione reductase and glutathione transferase [26, 27]. Heme oxygenase is a heat shock protein responsible for the production of the antioxidants biliverdin and bilirubin [28].

Sulfur containing amino acids (cysteine and methionine) are susceptible to oxidation. SH-groups of cysteine residues of some specific proteins, (such as the glucocorticoid receptor) are maintained in the reduced state or just inversely: oxidized by the thioredoxins in the cytoplasm [29].

Oxidized methionines can be reduced by special enzymes, the methionine sulfoxide reductases, MSRs [30–32]. An increasing number of evidence indicate that this system may serve as an additional antioxidant mechanism scavenging oxidative agents [33, 34]. The prokaryotic homologue of MSRs can be integrated into the outer membrane

Table 2

Cytoplasmic redox chaperones

Chaperone	Function	References
small heat shock proteins (Hsp25, Hsp27)	increases reduced glutathione levels by increased glucose-6-phosphate dehydrogenase, glutathione reductase and glutathione transferase activities	26, 27
Hsp32[a]	heme oxygenase-1 an important component of oxidative stress-mediated cell injury	28
Hsp33	oxidation-activated chaperone in yeast	69
Hsp70 cytochrome c	has (probably indirect) anti-oxidant properties released from mitochondria in apoptosis, chaperone function has been detected	8, 9, 70 36
thioredoxin[a]	promotes cytoplasmic oxidation of selected proteins	29
ERV1/ALR[a]	promotes the cytoplasmic formation of disulfide bridges	30
MsrA and MsrB[a]	methionine sulfoxide reductase: regenerates functional methionine	31

[a]No direct chaperone activity has been demonstrated yet.

helping the survival of the pathogen in the presence of reactive oxygen species secreted by the host [35].

An interesting member of redox cytoplasmic chaperones is cytochrome c, which is only a Ñguest„ in the cytoplasm during its apoptotic release from mitochondria and has been established as a chaperone long time ago [36].

CHANGES OF CELLULAR REDOX BALANCE IN DIABETES MELLITUS

The diabetes mellitus is described as a complex metabolic disease characterized by the absolute or relative shortage of insulin. One of the consequences of the metabolic disorganization is the increased generation of ROS [37, 38]. The oxidative stress is initially prevented by the different antioxidative defense systems, but later on these mechanisms become exhausted, and oxidative damage develops. The elevated concentration of the oxidative stressors is the result of the disorganized function of the mitochondria, the altered metabolism of the arachidonic acid, protein kinase C activation, glucose autooxidation, and the free radicals generated by the non-enzymatically glycated proteins. These redox changes are typical of the extracellular space, but the signs of the oxidative stress are also detectable in the intracellular space [39].

Besides the oxidative damages of the membranes and enzymes diabetic thiol metabolism might also be affected by other factors. There are several pieces of evidence showing that the intracellular level of ascorbic acid, tocopherol and FAD is lower in both diabetic animal models and diabetic patients [40]. These changes are caused partly because of the accelerated cofactor consumption, and partly by the deficient cofactor-transport. Both the plasma membrane transporter of ascorbic acid and dehydroascorbic acid and their microsomal uptake are blocked by high glucose concentration [41, 42]. The activity of some FAD-containing enzymes is significantly lower in experimental diabetes. These small molecules are all suggested to take part in the thiol metabolism of the ER, and more experiments are required to decide if they are responsible for the suspected disturbances of the redox state and protein folding. Decreased PDI amounts and functional changes of the enzyme were also observed in diabetes.

Redox changes in diabetes alter the structure and perhaps the function of the ER redox protein folding-machinery in liver causing a folding deficient state. This might be a possible explanation of the decreased hepatic protein secretion observed in some studies [43], and can also contribute to the accelerated protein turnover detected upon oxidative stress conditions [44]. The exact consequences of the more reducing ER environment are not clear yet. However, the regulatory role of the redox state is well known in many processes. PDI chaperone activity was found to be redox sensitive, where the accumulation of reduced PDI can lead the formation of more noncovalently bound complexes and a slowdown in redox-chaperoning [23]. Reducing conditions may also decrease protein stability and increase ER protein degradation. These redox changes in diabetes may influence the transport, presence and redox function of extracellular PDI on the plasma membrane [45]. The origin of the ER redox change is still unknown, but the alteration of the ERO1-L redox state in the process can enforce the role of the cytoplasmic redox changes typical of the disorganized metabolism in diabetes mellitus. The identification of the exact causes of these changes can help us to discover the specific cytosolic link of the ERO1-L mediated disulfide bond formation.

CHANGES OF CHAPERONE ACTIVITY AND PROTEIN FOLDING IN DIABETES

Elevated glucose levels also induce various proteotoxic damages in diabetes. Glycation, formation of advanced glycation endproducts,

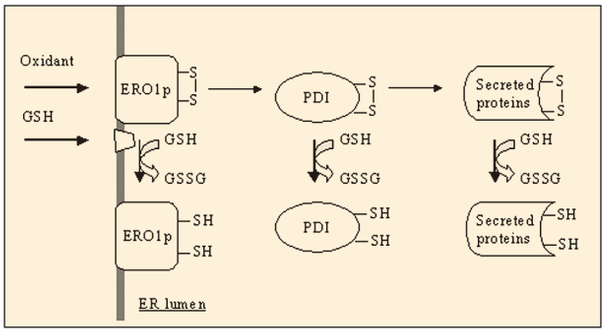

Figure 1

Redox balance in the ER

ERO1p, protein disulfide isomerase reoxidizing protein; PDI, protein disulfide isomerase; GSH, reduced glutathion; GSSG, oxidized glutathion; ER, endoplasmic reticulum. Adapted from ref. [25].

increased protein oxidation and other types of protein damage all occur. The increased amount of misfolded, nonfunctional proteins have higher ability to aggregate and are easy targets of the irreversible protein oxidation and carbonylation [46]. Besides these changes, the protein degradation machineries, such as the proteasome are also impaired. These effects all point towards an increased need for chaperone action to repair damaged proteins as well as the proteolytic apparatus.

There is a well established link between changes in extracellular glucose level and the regulation of the synthesis of several molecular chaperones, such as glucose-regulated proteins [9, 47, 48]. Moreover, numerous *in vitro* and *in vivo* results exist about the stimulation of heat shock protein synthesis and function by the elevated level of reactive oxygen species, non-physiological disulfide bridges and misfolded proteins. However, just a few reports explored the qualitative and quantitative changes of chaperone function in diabetes [49–53].

Increased level of Hsp25 and dissociation of the homooligomers was detected in kidney after streptozotocin induced diabetes. This alteration may influence the mesangial cell contractility in rats [54]. Another member of the family of small heat shock proteins, lens -crystallin, is able to save other proteins against nonenzymatic glycation. However, if the -crystallin itself becomes glycated, its chaperone activity is severly impaired [55, 56]. Another diabetic protein damage, glyoxylation also impairs the chaperone function of -crystallin [57], which may ìsacrifice„ itself to protect other enzymes, such as the Na/K-ATPase [58] from this damage. Interestingly, a recent study showed that methylglyoxal modification at low concentrations of the agent, just inversely, enhances the chaperone action of both -crystallin and the related Hsp27 [59].

Synthesis of the 60, 70 and 90 kDa heat shock proteins is also very sensitive to the oxidative stress by different stressors (ethanol, oxidized low density lipoprotein, sulfhydryl oxidants, reactive oxygen species etc.), but the characteristics of heat shock protein induction is not clear at all under chronic diabetes. Controversial effects of diabetes on the expression of Hsp70 were observed in some experiments. While levels of the constitutively expressed Hsc70 were reported to decrease in the livers of STZ-rats, other organs did not show this effect and levels of other chaperones were unchanged [53]. A correlation was found between the decreased expression of the inducible Hsp70 in skeletal muscle and the insulin resistance in diabetic patients [49]. While an increase of Hsp70 was reported from mononuclear cells of diabetic patients [51], a general decrease of Hsp70 inducibility was also observed [53, 60]. The extent of protein damage and the duration of the disease may both affect the level and inducibility of all chaperones, which may in part explain the controversial results.

Despite of the differences in chaperone induction at various chaperone classes, the protective effect of chaperones on diabetes-induced

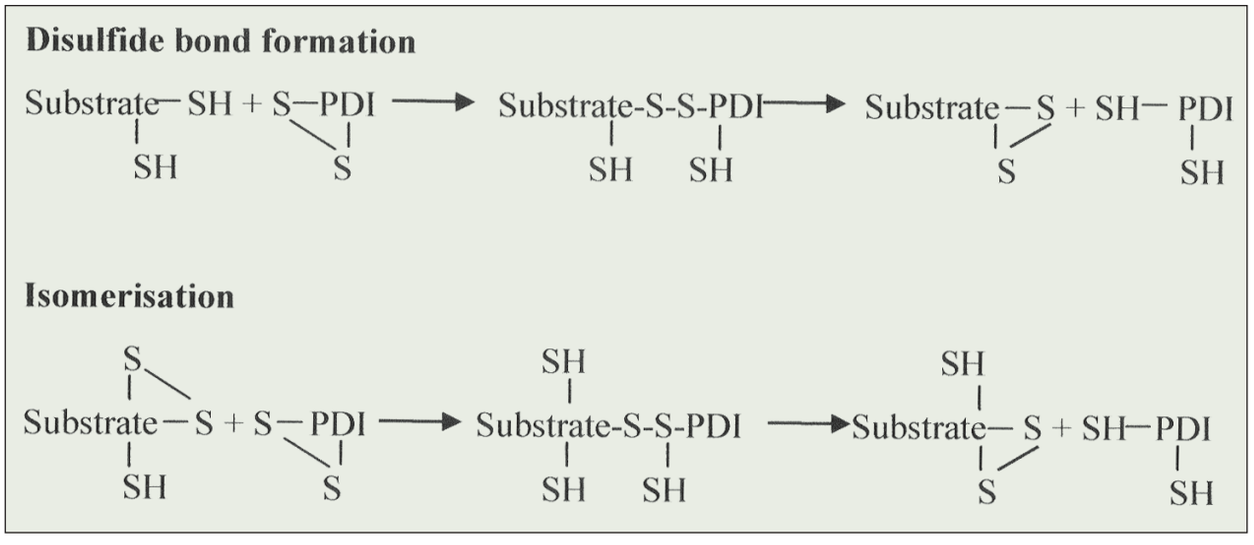

Figure 2

The mechanisms of thiol-disulfide exchange

PDI, protein disulfide isomerase.

protein damage seems to be fairly general. As a sign of this, similarly to the effect of -crystallin on Na/K-ATPase [58], the bacterial homologue of Hsp60, GroEL, delays the inactivation of glucose-6-phosphate dehydrogenase by nonenzymatic glycation [55].

The protecting effects of chaperones were utilized in the development of a recent class of pharmaceutical agents able to help chaperone induction after an initial stress [61]. These chaperone co-inducer drug candidates were able improve both peripheral neuropathy [62, 63] and retinopathy [64] in diabetic animal models. Chaperones may become a novel class of highly efficient pharmaceutical targets of diabetes medicine in the future.

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