PROTEIN FOLDING IN DIABETES

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Procesul de pliere a proteinelor *in vivo* are numeroase etape, care necesită o anumită asistență. Aşa-numitele „molecular chaperones" sunt necesare multor proteine pentru a se plia în cadrul unor structuri native şi pentru transportarea lor către destinația finală din cadrul celulei. Cele mai multe dintre proteinele de fază acută şi proteinele legate de metabolismul glucozei sunt „chaperones", care formează un sistem primordial de autoapărare intracelulară. „Molecular chaperones" au o importanţă profundă în practica medicală. Funcția lor este necesară pentru homeostazia celulei vii şi devine cu atât mai însemnată în patologie, cu cât celulele noastre trebuie să facă față unor împrejurări neprielnice. O perturbare proteotoxică este larg răspândită în bolile cronice, cum este diabetul. Cercetări recente în domeniul respectiv au arătat că îmbunătățirea funcției de „chaperone" poate întârzia sau diminua complicațiile cronice ale diabetului, cum ar fi angiopatia, neuropatia sau retinopatia. Capitolul de față sintetizează cunoştințele actuale privind procesul de pliere şi „molecular chaperones" în diabet, incluzând unele rezultate noi de laborator ale autorilor.

INTRODUCTION

Protein folding has numerous steps, which need assistance *in vivo*. Molecular chaperones are required for many proteins to fold, or re-fold into native structures, for their oligomeric assembly, and transport to their final destination inside the cell. Most of the heat shock proteins and glucose regulated proteins are chaperones forming an ancient, primary system for "intracellular self-defense" [1,2]. Molecular chaperones have a profound importance in medical practice. Their function is necessary for the homeostasis of the living cell, and becomes especially important in disease, when our cells have to cope with a stressful environment [3,4]. Proteotoxic damage becomes especially widespread in chronic diseases, such as diabetes. Recent advances in the field proved that enhancement of chaperone function may postpone and/or diminish the chronic consequences of diabetes, like angiopathy, neuropathy and retinopathy [5–8].

PROTEIN FOLDING - AN ASSISTED PROCESS

Protein folding is characterized by three major steps *in vitro* (Figure 1) [9–14]. In the *first few milliseconds* most of the secondary structure is already formed. In most cases folding starts with the formation of alpha-helices, since here the participation of only adjacent amino acids is required. Beta-sheet formation establishes H-bonds between amino acids, which are far from each other in the primary sequence, therefore a greater decrease of entropy occurs than in the formation of alpha-helices. In the end of this first step the unfolded protein is collapsed, and a (more-less) stable intermedier, the molten globule is formed.

The partially folded state of *molten globule*s can be characterized by a developed secondary structure, which is mostly un-organized showing almost no tertiary structure [11–13]. Molten globules still have large unburied hydrophobic surfaces, therefore are subjects of extensive aggregation. The volume of molten globules, however, is almost as small as that of the final, folded protein.

The *last steps* of protein folding are the slow, rate-limiting steps [9,10]. Here the inner, hydrophobic core of the protein is organized [14] and unique, high-energy bonds are formed, such as disulfide bridges, ion-pairs, and the isomerization of proline *cis/trans* peptide bonds occurs. The free energy gain of these processes enables the formation of local, thermodynamically unstable, "high-energy" protein structures, which are stabilized by thermodynamically favorable conformation of the rest (bulk) of the protein. These "high-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.

Protein folding is not a straightforward process. Dead-end streets, reverse reactions, futile cycles are all characteristic to it. A minor amount of fully folded, native protein always co-exists with various forms of molten globules and with traces of remaining unfolded species. This unordered, hesitating, zigzag flow of events needs a lot of help. 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. They recognize and cover hydrophobic surfaces successfully competing with the aggregation process.

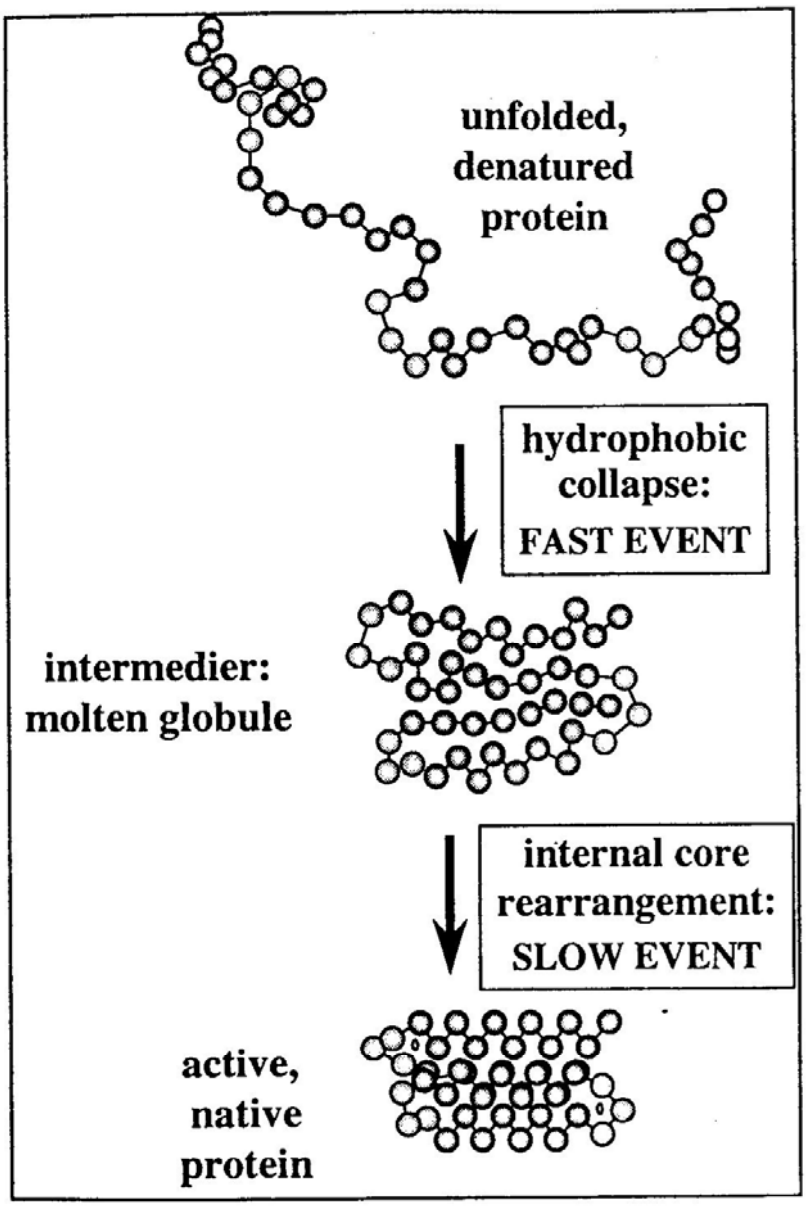

Figure 1. Major steps of protein folding *in vitro* [14].

Unaided protein folding can be very slow. Thanks God, the high probability of alpha-helix formation from adjacent amino acids, and the autocatalysis of the folding process, is not as slow as it was predicted by the simple mathematics of the Levinthal paradox [15], since this way the lifetime of the whole universe would not be enough to allow the formation of even the simplest protein. We need a faster speed. The slow rate limiting steps have to be accelerated. Rearrangement of the hydrophobic core is aided by periodic pulling and water-percolation [14] and the "traditional" molecular chaperones may also provide better circumstances for ion-pair formation and for the establishment of "high-energy" protein segments. Disulfide bridge formation and proline *cis/trans* isomerization are promoted by protein disulfide isomerases and by peptidyl-prolyl-*cis/trans* isomerases, respectively [16,17].

Molecular chaperones recognize hydrophobic surfaces and – most probably – peptide bonds protruding to the surface of the protein. Folded proteins keep their hydrophobic amino acids in their interior and peptide bonds are made inaccessible by the covering amino acid side chains in established secondary structures; such as in alpha-helices, or in beta-sheets.

Chaperones may aid protein folding in a direct way, by facilitating the transformation of unfolded species to the fully folded protein. However, they may also unfold the incorrectly folded proteins, thus giving them another chance for spontaneous refolding [1,2,18]. These two processes may go in parallel, or may be characteristic to different chaperon/target pairs.

Nascent proteins have the unique situation that they have to fold, when they are not even ready yet. The first protein segment, which leaves the ribosome, surely has an energy minimum different from the whole protein. In many cases *in vivo* protein folding has to be delayed. Molecular chaperones are attached to the ribosomes "waiting" for the nascent protein chain. When it appears, the chaperones "sit on it" preventing premature protein folding before the rest of the protein is synthesized [19,20].

Chaperones direct proteins inside the cell. Pores of the mitochondria or of the endoplasmic reticulum are too small to accommodate fully folded, globular proteins. Proteins have to unfold to get through, and to re-fold in the lumen of the organelle [21].

Molecular chaperones not only help, but also destroy. Some incorrectly folded proteins – maybe those which have lost not only their tertiary, but also their secondary structure leaving their peptide bonds accessible – are presented to the lysosomal protein degradation [22] or to the extralysosomal proteasome [23]. In case of massive protein damage, when the amount of degradable proteins exceeds the capacity of the intracellular proteolytic systems, chaperones help to form inclusion bodies to seggregate damaged proteins [24].

MOLECULAR CHAPERONES IN MEDICINE

Molecular chaperones are one of the most conserved proteins in living organisms [1,2]. Invading bacteria experience major changes in their environment when entering their host. These changes and the defense mechanisms (depletion of nutrients, pH changes, digestive enzymes, peroxides, superoxides and an increase in temperature) induce numerous molecular chaperones in the bacteria, among which some are also expressed on the bacterial surface. Because of their conservative structure, these bacterial

heat shock proteins, especially Hsp65, give a common recognition signal, and therefore provoke a general, high-capacity immune response [1–4].

In some unfortunate cases (such as in *rheumatoid arthritis* and in *insulin dependent diabetes mellitus* -IDDM-) certain proteins of the host organism resemble some epitopes of these bacterial heat shock proteins. In these patients the common, antibacterial immune response attacks the cells bearing these host-proteins, and a severe autoimmune response develops. "Vaccination" with modified epitopes of bacterial Hsp65 diminish and in some cases prevent the development of arthritis or IDDM [25,26].

Certain molecular chaperones, especially Hsp70, Hsp90 and Grp94, bind to various peptides besides their protein targets. These chaperones behave as tumor-specific transplantation antigens, i.e. immunization with these chaperones isolated from a certain tumor provokes a tumor-specific immune response and prevents the spread of the original tumor in the vaccinated animal. Investigation of the molecular mechanism of this surprising phenomenon cleared that not the chaperones *per se* are the immunogens, but their complexes with the tumor-derived peptides. These chaperones form a "relay-system", which presents various peptides of the host cell to the MHC-I complex enabling an efficient cytotoxic immune response. However, due to the extreme conservativity of chaperones, "foreign" chaperones "filled" with foreign peptides may also enter this pathway leading to "cross-priming", i.e. the presentation of foreign peptides by the MHC-I complex instead of MHC-II. Thus, an efficient cytotoxic immune response could be generated for foreign peptides, which provides an excellent tool for vaccination against various viral infections and cancer [27].

Molecular chaperones are responsible for the "conformational homeostasis" of cellular proteins. When the homeostasis of the host organism is perturbed, an increased capacity of the "chaperone machines" is highly advantageous. Many of the perturbations (such as alcohol, other poisons, sunburn, anxiety, etc.) may induce the synthesis of these chaperone proteins *per se*, but in case of bacterial and viral infections the developing fever also helps this process. Ischemia and the consecutive oxidative damage of reperfusion are also common environmental perturbations in higher organisms. Since Currie *et al.* [28] have shown that the induction of molecular chaperones, most notably Hsp70, may prevent the cardiac muscle from the damage of both ischemia and reperfusion, molecular chaperones are actively investigated as possible tools in the treatment of heart attack or stroke. Their protective role is also used in organ transplantation, where a

prior heat treatment induces a more efficient organ-survival and diminishes the occurrence of rejection by the host organism. The quality control of endoplasmic reticulum chaperones aggravates several protein folding diseases such as cystic fibrosis, pulmonary emphysema or diseases of lipid metabolism and protein glycosylation. Heat shock proteins are major constituents of inclusion bodies characteristic to most of the autodegenerative diseases, such as the Alzheimer disease, Parkinson disease, cirrhosis and the Creutzfeld-Jakob syndrome. Chaperone-induction can be used as an efficient monitoring system for environmental damage [2–4].

Aging is frequently described as a consequence of an impaired function of repair processes (immune system, DNA-repair, elimination of free radicals, etc.). Molecular chaperone-catalyzed refolding of damaged proteins may well be one of these crucial repair processes. In agreement with this hypothesis, aged organisms contain an increased amount of misfolded proteins, and the induction of Hsp70 is impaired in both aged rats and humans. Moreover, chaperone induction increases the life expectancy leading to a significant improvement in longevity of the affected animals [2,29,30].

PROTEIN FOLDING AND CHAPERONE ACTION IN DIABETES

Elevated glucose levels induce various proteotoxic damages in diabetes. Glycation, formation of advanced glycation endproducts, increased protein oxidation and other types of protein damage all occur. The increased amount of misfolded, nonfunctional proteins have a higher ability to aggregate and are easy targets of the irreversible protein oxidation and carbonylation [30]. 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 [2-4]. 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 action *per se* in diabetes [31–34].

Increased level of Hsp25 and dissociation of the homooligomers were detected in the kidney after streptozotocin induced diabetes. This alteration may influence the mesangial cell contractility in rats [35]. Another member of the family of small heat shock proteins, lens α-crystallin, is able to save other proteins against nonenzymatic glycation. However, if the α-crystallin itself becomes glycated, its chaperone activity is severely impaired [36,37].

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 are not clear at all under chronic diabetes. The bacterial homologue of Hsp60, GroEL, delays the inactivation of glucose-6-phosphate dehydrogenase by nonenzymatic glycation [36]. Controversial effects of diabetes on the expression of Hsp70 were observed in some experiments [33,38].

The endoplasmic reticulum (ER) is a central organelle of chaperone action. Here, classical molecular chaperones and protein folding catalysts (i.e.: protein disulfide isomerases, peptidyl-prolyl isomerases) help not only the transport of the newly synthesized proteins through the ER membrane, but to acquire the final structure of different glycoproteins and the folding and assembly of secreted and membrane proteins [39]. Experimental data show that the total protein secretion and the level of some specific secreted proteins are decreased in diabetes [40,41], but the behavior of ER-chaperones is rather unclear. Changes of Grp78 were detected by different authors, and a decrease of the level [32,34] and activity of protein disulfide isomerase in microsome preparations isolated from rat liver (Figure 2) was also established.

Recent reports showed that the phase-II drug candidate, chaperone-coinducer Bimoclomol, is a potent compound inducing an acceleration of diabetic wound-healing and slowing diabetic neuropathy [5–7]. Our earlier studies demonstrated that another well-established chaperone-inducer, the arginine analogue canavaline, is also able to diminish streptozotocin-diabetes induced retinopathy [8].

In summary, changes of chaperones in diabetes are not fully analogous with those under different, acute stress conditions. This may be explained by the compensatory effects of other antioxidant systems and/or the positive or negative changes in the complex regulation of heat shock protein synthesis and activity. Clearly we need further studies to explore the role of chaperones in the pathomechanism of diabetes mellitus and find their possible use in the clinical treatment of this disease.

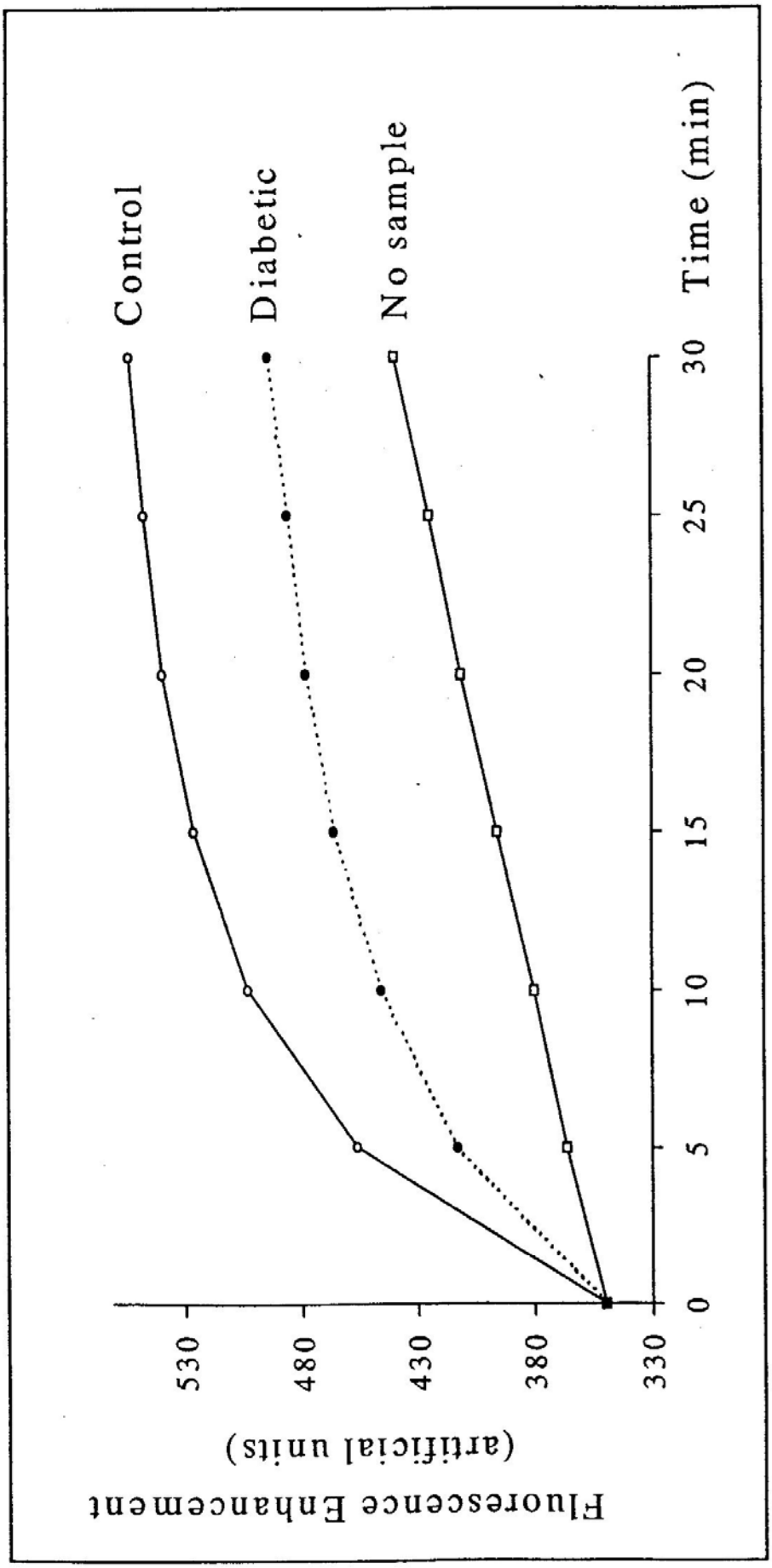

Figure 2. Protein disulfide isomerase activity of liver microsomes isolated from control and streptozotocin-diabetic rats. *Wistar rats were treated with 65 mg/kg i.v. streptozotocin and sacrificed 4 weeks after the treatment. Microsome isolation was immediately performed according to Lambert and Freedman (46). The protein disulfide isomerase activity was measured by a new fluorescent assay (47), where the aggregation of 0.7 μM fluorescently labelled insulin was followed in the absence (open square) and in the presence of 100 mg/ml control (open circle) and diabetic (filled circle) microsomes. Data are extracted from three identical experiments.*

POTENTIAL CLINICAL ASSAYS FOR CHAPERONE ACTION

The paramount importance of molecular chaperones in recovery from various diseases necessitates the monitoring of their activity in patients. May well be that the "chaperone-status" will be a common marker of health in the near future. Unfortunately, until recently we did not have easy methods to determine chaperone-related activities in whole cellular homogenates. However, recent progress in the biochemistry of molecular chaperones enables us to construct and try such methods.

Chaperone activity of whole cell homogenates may be assessed using several test systems such as the luciferase-renaturation assay [42]. To check the chaperone activity of purified chaperones and chaperone-complexes the citrate synthase-assay is also available [43].

Autophosphorylation in the presence of Ca-ATP is a common feature of almost all molecular chaperones [44]. Since most of protein kinases cannot utilize Ca-ATP, phosphorylation of cellular proteins in the presence of Ca-ATP gives a surprisingly clear pattern. In streptozotocin-diabetic rats the phosphorylation of an approx. 94 kDa protein is diminished [45]. Insulin-treatment reverses the effect. Earlier we have established that this protein is the 94 kDa glucose-regulated protein, Grp94 [44].

Unfortunately, the *ATPase* reaction is not so specific as the autophosphorylation. Therefore, specific measurement of chaperone/ATPases may only be accomplished by using specific inhibitors of ATPase activity, such as geldanamycin for Hsp90 [44].

SUMMARY AND PERSPECTIVES

In recent years we have learned a lot about the molecular mechanism of protein folding *in vitro* and some important features were also revealed of the *in vivo* formation and repair of protein structure. Many aspects of the molecular mechanism of chaperone action were cleared, and we also recognized the importance of these proteins in the clinical practice. Chaperones are essential to prevent the proteotoxic damage in chronic diseases such as diabetes. Nevertheless, relatively few is known about the changes of chaperone action in this disease and the therapeutic potential of chaperone inducers as anti-diabetic drugs is also largely unexplored. We hope that with this short review we may increase the courage of some fellow scientists to enter this difficult, but very promising path of clinical research.

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