Protein Misfolding in Conformational Disorders: Rescue of Folding Defects and Chemical Chaperoning

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Abstract: Protein folding in the cell is a tightly regulated process, involving a series of proteins, from molecular chaperones to proteases that assist the folding process and monitor the quality of the final product. Despite this control, genetic or sporadic factors may compromise protein folding and the folded state resulting in the formation of non-native misfolded, destabilised, aggregated or fibrillar species. These are hallmarks of the so-called protein conformational disorders, in which the altered protein conformations result in cell toxicity, functional deficiency or lead to dominant negative effects. Examples of such pathologies include neurodegenerative and metabolic disorders. In recent years, it has become clear that several different small chemical compounds such as osmolytes, protein inhibitors, ligands and cofactors exert a chemical chaperoning effect and are able to rescue folding and trafficking defects, minimising or partly overcoming the pathological consequences of protein misfolding. Here we review the different types of chemical chaperones and provide a structural and energetic rationale for their action. Examples of chemical chaperoning are overviewed and discussed on the basis of the reported effects exerted by chemical compounds at different stages of the protein folding process and protein conformational states.

Key Words: Chemical chaperones, pharmacological chaperones, misfolding, protein folding, human disease.

1. INTRODUCTION

Several human diseases are caused by defects in protein folding, which may result from inborn or acquired genetic errors, or as a result of sporadic factors such as adverse intracellular conditions [1]. Examples of such pathologies which have been designated as conformational disorders, include Alzheimer's (AD) and Parkinson's disease (PD), spongiform encephalopaties, familial amyloid polyneuropathy (FAP) and several metabolic disorders such as phenylketonuria (PKU) and fatty acid oxidation defects, among many others [1,2]. Misfolded proteins exert a pathological effect since due to its conformational changes, they may present a deficient biological function e.g. [3], undergo an accelerated degradation or accumulate as toxic aggregates [4]. Possible therapeutic strategies for protein misfolding diseases aim to rescue the native protein conformation or to induce the stabilisation of intermediate or misfolded protein states. With this objective, in recent years small molecules referred to as chemical and pharmacological chaperones have been employed to rescue protein folding defects. In this review we will discuss the mode of action of these compounds in the context of protein folding and misfolding in the cellular environment, with an emphasis on the energetics and mechanistic mode of action of these small molecules. A particular case study covering our investigations on the stabilisation by osmolytes of PKU-related phenylalanine hydroxylase (PAH) mutations will also be covered.

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2. PROTEIN FOLDING AND PATHOLOGICAL NON-NATIVE CONFORMATIONS

Protein Folding in the Cell

The majority of the biologically active proteins must present a specific three-dimensional structure, which is determined by its amino acid sequence. The folding of a protein to its functional conformation builds upon a set of interactions established between a limited number of specific residues which nucleate and trigger the cooperative folding reaction, into the final topology. Particular folding pathways, rather than a random search of conformations, determine the folding landscape of a protein which is funnelled towards an energetic minimum corresponding to the most stable structure under physiological conditions [4]. This process which occurs spontaneously for small polypeptides (~100 residues), requires assistance for the proper folding of larger polypeptides or protein domains on a milliseconds-to-seconds time scale. Hence, cells developed a tightly regulated pathway which includes folding catalysts (e.g. cofactors) and protein molecular chaperones, such as the heat shock proteins, trigger factor and chaperonins [5,6]. These components of the protein folding machinery do not determine the folding trajectory into any particular pathway, as this information is solely contained in the protein chain itself [7]. Rather, correct folding is promoted essentially by shielding hydrophobic patches and unstructured regions which become exposed during translation in a cellular environment where confinement and crowding effects are determinant for the protein folding reaction. Confinement is thought to favour the folded state and increase folding stability as the number of explored conformations become restricted and nucleating contacts are

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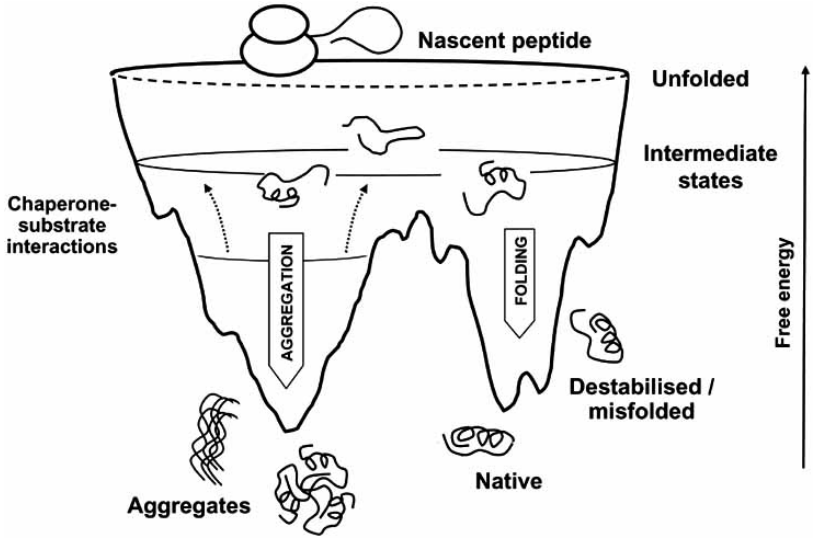

Fig. (1). Energetic funnel representing protein folding events in the cell.

Upon ribosomal biosynthesis a large ensemble of random conformations is produced from which partially folded intermediate states are formed. Interaction of the latter with molecular chaperones leads to folding into a native structure. Concomitant misfolding and aggregation pathways are also available (left), and upon chaperone association-dissociation interactions the free energy of these states is raised, enabling productive folding to occur (right). Mutations or adverse physiological conditions may trap destabilised/misfolded conformations either at low free energy depressions or at a point from which aggregates could also be formed. Adapted from [1,77].

favoured. This is what happens inside chaperonin complexes and in the exit tunnel of the ribosome (10-100 Å), as positions in which part of the protein chain lies outside the walls cannot be sampled. In contrast, the very high cellular concentration of macromolecules in the cell (300-400 $mg.ml^{-1}$ in *Escherichia coli* [8]) results in crowding effects which contribute to a lower efficiency and quality of protein folding, as non-native interactions are promoted. The association with chaperone proteins minimizes both aggregation prone conformations and other unstable misfolded conformations. Further supervision of all synthesised proteins involves a series of other components which constitute the protein quality control (PQC) system [1,6]. Among these are for example proteases which eliminate misfolded proteins by proteolysis. Nevertheless, this folding machinery is not fail proof and genetic defects and adverse cellular or physiological factors may compromise correct protein folding, resulting in non-native protein conformations to occur in the cell [1].

Disease Prone Protein Conformations

Protein misfolding and protein folding defects are intrinsic to the protein folding reaction, as a polypeptide chain can be converted into different types of non-native structures. However, amino acid alterations or adverse sporadic factors may cause other non-native conformational states to be accessible. In the above mentioned perspective of the folding funnel, this results in detours from the folding pathways, yielding either dead-end conformations or non-productive pathways to be favoured, Fig. (**1**). The cellular consequences of these effects are multifold and have been comprehensively covered [1,9,10]. For example, since the PQC system has the capacity to recognize non-native protein states and rapidly degrade them, this would lead to a clearance of misfolded polypeptides, diminishing the intracellular protein levels, resulting in a loss-of-function. However, sometimes the stable misfolded conformations overload the chaperone system, or have a tendency to aggregate, resulting in toxic cellular effects. The latter is indeed the case of amyloid fibrils and plaques, which are particularly relevant in the context of a vast number of clinical syndromes that involve amyloid deposition [2]. Several recent excellent reviews have provided an extensive coverage on amyloid-type diseases and on the mechanisms of fibril formation at a molecular level [2,4,11,12]. However, not all conformational disorders involve protein aggregation or amyloid deposition. About one half of all sequence alterations in genetic diseases are missense mutations [13], and the effect of these modifications frequently affects the structure, stability and folding of the mutated proteins. Many of these conformational effects do not yield an amyloid-precursor state, but rather result into slightly misfolded or conformationally destabilised proteins (C.f. examples in Table **1**). In these cases, the amount of mutant protein in the cell will be decreased to a level that depends on the balance between its folding and degradation [13]. This is reflected by the fact that many loss-of-function disorders have phenotypes ranging from mild to severe. A mild phenotype is suggestive that some functional protein is nevertheless available in the patient cell; adverse cellular conditions such as an increase in temperature as a result of fever increases the misfolded fraction shifting the balance to degradation [13].

3. CHEMICAL CHAPERONING

Rescue of Protein Folding Defects and Cellular Mislocalization by Small Chemicals

In recent years the use of small molecules to rescue folding defects in proteins involved in conformational disorders has been explored and overviewed in several reviews [14-19]. Chemically induced stabilisation results essentially from

Table 1. Human Proteins Involved in Misfolded Disorders Rescued by Chemical and Pharmacological Chaperones

Disease	Protein Involved	Functional Localization	Mechanism of Pathogenesis	Chemical Chaperone/ Pharmacological Chaperone
Gaucher	GC	Lysosome	Mistrafficking	Deoxynojirimycin derivatives [24]
Fabry	GLA	Lysosome	Mistrafficking	Galactose [43] 1-deoxy-galactonojirimycin [44]
Pompe	GAA	Lysosome	Mistrafficking	Deoxynojirimycin derivatives [78]
Tay-Sachs	HEXA	Lysosome	Mistrafficking	PYR; NGT [79]
Menkes	MNK	Membrane	Mistrafficking	Copper [53]
Familial hypercholesterolemia	LDL receptor	Membrane	Mistrafficking	4-PB [80]
Cystic fibrosis	CFTR	Membrane	Mistrafficking	Glycerol, DMSO,TMAO [47] 4-PB [81] TS3 (limonoid compound) [49] VRT325 [51] VRT325; corr-2b; corr-4a [52]
Nephrogenic diabetes insipidus X-linked	V2R	Membrane	Mistrafficking	SR121463B [82]
Nephrogenic diabetes insipidus type II	AQP2	Membrane	Mistrafficking	Glycerol; TMAO; DMSO [83]
α1-antitripsin deficiency	α1-AT	Extracellular	Mistrafficking	Glycerol, 4-PB [54]
Retinitis pigmentosa	CA IV	Membrane	Mistrafficking	Acetazolamide; ethoxzolamide (enzyme inhibitors) [84]
Primary carnitine deficiency	OCTN2	Membrane	Mistrafficking	4-PB, quinidine, verapamil
Albinism	Tyrosinase	Membrane	Mistrafficking	DOPA, Tyr [85]
Huntington	Huntingtin	Cytoplasm	Aggregation	Trehalose [58]
Hypogonadotroic hypogonadism	GnRH receptor	Membrane	Mistrafficking	Indol and Quinolone derivatives [86]
Machado-Joseph	Ataxin-3	Nucleous/ cytoplasm	Aggregation	Glycerol, TMAO, DMSO [87]
Parkinson	α-synuclein	Cytoplasm	Misfolding/aggregation	TMAO [88]
Creutzfeldt-Jakob	Prion	Several cellular locali.	Aggregation	Acridine-based analogue [89,90]
Alzheimer	β-amyloid		Misfolding/aggregation	TMAO, glycerol [59]
Familial amyloid polyneuropathy	TTR	Extracellular	Aggregation	Thyroxin, TTR ligands [37,38]
Homocystinuria	CBS	Cytoplasm	Misfolding	TMAO, glycerol, sorbitol, proline; DMSO [33]
Phenylketonuria	PAH	Cytoplasm	Misfolding	Glycerol [75] BH_4 [72]
Maple syrup urine disease	BCKD	Mitochondria	Misfolding	TMAO [34]
Cancer	p53	Nucleus	Misfolding	Glycerol, TMAO, D_2O [64] CP249175, CP31398 [91]

(GC) glucocerebrosidase; (GLA) α-galactosidase A; (GAA) Acid α-glucosidase; (HEXA) β-hexosaminidase A; (MNK) Menkes protein; (CFTR) cystic fibrosis transmembrane conductance regulator; (α1-AT) α1-antitripsin; (TTR) transthyretin; (CBS) cistathionine-β-synthase; (BCKD) branched chain α-ketoacid decarboxylase, (V2R) vasopressin V2 receptor; (AQP2) aquaporin-2; (LDL) low density lipoprotein; (CA IV) carbonic anhydrase IV; (PAH) phenylalanine hydroxylase; (PYR) primethamine; (OCTN2) organic cation/carnitine transporter; (NGT) N-acetyl glucosamine thiazoline; (VRT-325) quinazoline compound; (corr-2b) thiazole compound; (corr-4a) bisaminomethylbithiazole compound.

a direct effect of small molecular weight compounds that correct protein misfolding or folding defects, or from a direct regulation[20] or substitution [21] of the folding activities of molecular chaperones. This effect is frequently relevant in the context of a functional deficiency resulting from a genetic anomaly or an adverse physiological condition, as even if only a minor fraction of the perturbed proteins undergo refolding or stabilisation. In fact, this may be enough to overcome the threshold of minimal activity required to recover biological function [22]. In some particular cases, chemical chaperoning is likely to lead to a concrete therapeutic use, especially in lysosomal storage disorders, such as Gaucher's disease [23,24]. The type of molecules that are able to exert this effect is diverse, Fig. (**2**), and two broad categories can be defined, on the basis of their mode and mechanism of action. Some authors have grouped these compounds as chemical and pharmacological chaperones, essentially to distinguish between the unspecific action of the former and the more specific direct action over a particular target protein observed in the latter. Independently of the nomenclatures used, the generic overall effect results from chemically-mediated protein stabilisation or refolding, and in this respect, the term chemical chaperoning is in our opinion more comprehensive and will be hereon used to refer to the generality of these compounds.

Chemical Chaperoning by Osmolytes and Protein Stabilisers

One group of molecules comprises mainly osmolytes and protein stabilisers; this class of compounds, includes polyols (e.g. glycerol, sorbitol), sugars (e.g. trehalose), methylamines (e.g. trimethylamine-N-oxide, glycine, betaine and glycerophosphorylcholine) or even free aminoacids (e.g. glycine, taurin) or its derivatives (e.g. ectoine and gama-aminobutyric acid). Many of these compounds are synthesized or uptake by living organisms, from micro-organisms to plants and animals, to minimize protein denaturation as a result of adaptation to a harsh environment [25] or stress [26]. These molecules are frequently referred to as compatible solutes, considering that they can accumulate inside the cell without affecting the function of other macromolecules. Other low molecular weight compounds such as deuterium oxide (D_2O), dimethyl sulfoxide (DMSO) and 4-phenylbutyrate (4-PB) have also been associated to a chaperone-like function. The mechanism by which osmolytes promote protein folding and increase thermodynamic stability has been the focus of intense investigation. These compounds have an unspecific mode of action, and do not bind directly to proteins. Their action results from the hydration effect, which results from the ability of water molecules to establish favourable interactions with polar groups from the protein backbone, thus increasing protein compactness [27,28] , Fig. (**3**). This effect narrows the population of conformations in the vicinity of the native state configuration, by favouring folding-promoting contacts. Also, during protein synthesis, these compounds minimize the formation of intermediate states that might lead to folding dead-ends.

Chemical Chaperoning by Ligands, Inhibitors and Co-factors

Recently it has been shown that low molecular weight compounds which bind reversibly to a specific protein are

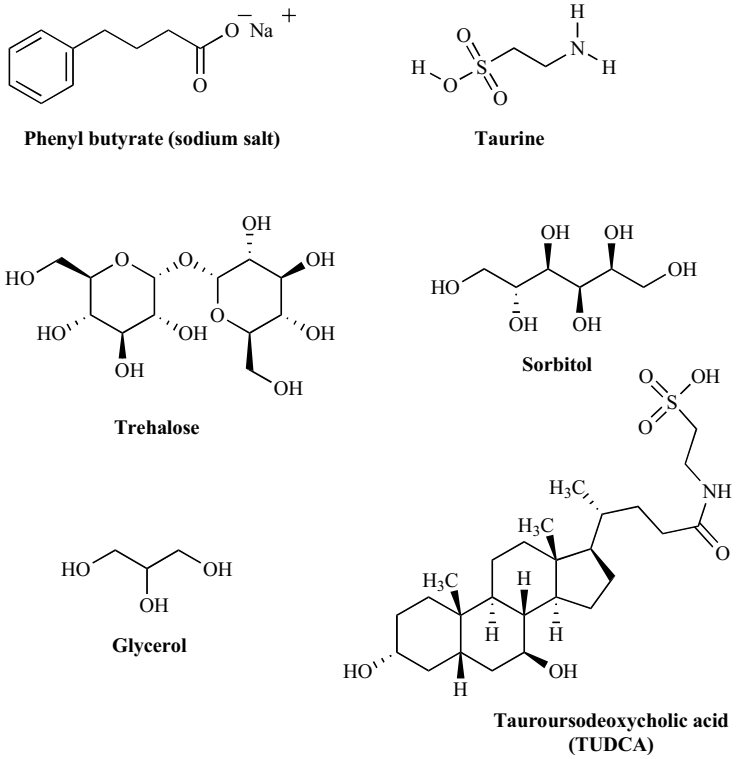

Fig. (2). Chemical structures of molecules with chemical chaperoning effect.

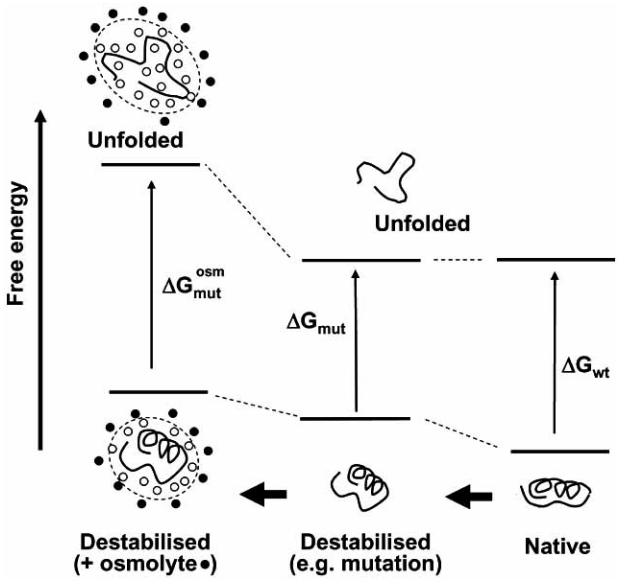

Fig. (3). Scheme depicting the effect of preferential hydration on protein stability.

The unfavourable interaction of the polypeptide with osmolytes (●) promotes preferential protein hydration by water molecules (○) resulting in an increase of the free energy of both the native and the unfolded state. In the depicted, the energy raise is more significant in the unfolded state, as a result of the increased protein surface area exposed upon unfolding [19,28].

also able to restore protein function and folding. As mentioned above, considering the specificity of action they have been classified as pharmacological chaperones [16,18,29]. This group is more heterogeneous as it comprises molecules that bind weakly to a specific target protein, and include competitive inhibitors, ligands, agonists/antagonists, and protein cofactors, including metal ions. Typically, these compounds either induce protein refolding or stabilisation, or contribute to structuring a particular region or domain within a protein. In fact, interactions with cofactors, either covalent or non-covalent bonds, are among those that also contribute significantly to the maintenance of the tertiary structure of a protein [30]. Proteins in the folded state have an inherent flexibility which is directly related to the fact that the net free energy of stabilisation is rather small, ranging from 20-40 $kJ.mol^{-1}$. The intrinsic breathing properties of a protein can become more enhanced whenever a ligand or a cofactor is absent. In this case, protein destabilisation may arise from the direct loss of a stabilizing interaction at the ligand or cofactor site, which increases the number of possible conformations accessible at that energetic stage; this may result in protein destabilisation or ultimately in protein misfolding. An extreme example is found in zinc finger domains, which unfold upon removal of the zinc ion [31].

In the context of disease-related protein misfolding, when the binding of a ligand or cofactor to the protein has an energetic contribution favouring the native state, then a chemical chaperoning effect is being effectively exerted by the chemical agent, Fig. (**4**). This may result from an interaction at an active site (for example, an inhibitor associating to a catalytic centre of an enzyme) or at a superficial or interfacial region of a protein (for example association of an organic cofactor). The energetic favourable contribution may directly result from an enthalpic gain arising from the new interactions, or from a change in quaternary structure, if binding of a ligand or cofactor favours more stable oligomeric states.

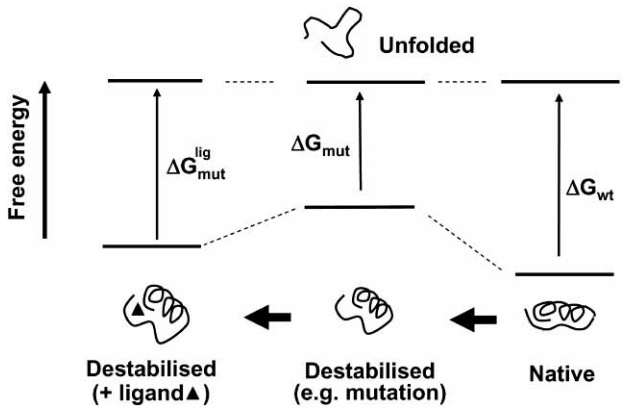

Fig. (4). Scheme illustrating a possible effect of a ligand on protein stability.

In this diagram, the binding of a chemical ligand (▲, e.g. inhibitor, cofactor, etc) has an effect on a destabilised protein variant, yielding a lower energy conformation.

4. AN OVERVIEW OF CHEMICAL CHAPERONE INTERVENTION

As previously discussed, the conformational states which are accessible to a polypeptide are rather diverse. In the context of a genetic mutation or adverse cellular condition, the probability of having increased populations of non-native states is substantially higher, among destabilised conformations, misfolded forms, aggregates, fibres or amyloid fibrils. In recent years, the combined observations from several excellent studies have shown that chemical chaperones can intervene at different stages of the protein folding process. For example, either by directing the folding pathways avoiding dead end intermediate states; by acting directly over the native conformation; stabilising it and preventing aggregation; or, by promoting refolding of misfolded or destabilised variants. These effects, which are not restricted to a particular cell compartment but have been associated both to cytosolic and endoplasmic reticulum (ER) folding, allow restoring function, as a consequence of structural stabilisation of the protein conformation, or correction of trafficking defects. Rather than describing how a particular protein and associated pathology can benefit from chemical chaperoning, we will illustrate the diversity of modes of action of several chemicals by discussing a few cases in which low molecular weight compounds are shown to play a role at different stages of the protein folding process and trafficking. However, it should be noted that chemical chaperones have a very broad palette of effects and that one molecule may have a

transversal effect, exerting its action on the folding of a protein, on the stabilisation of a particular conformational state, or in the modulation of the protein quality control pathways.

Effect on Folding Mechanism and Pathways

Chemical chaperones have been shown to play a role in determining the folding mechanism of proteins. One study providing direct evidence for this effect resulted from an investigation of the effect of TMAO and sarcosine on the folding of a model prokaryotic protein, barstar [32]. It has been showed that during its folding, barstar presents an ensemble of intermediate folding states which are structurally heterogeneous. In the presence of TMAO or sarcosine, these became more homogeneous and structured. Therefore, it has been postulated that osmolytes effectively reduce unproductive folding pathways, by thermodynamically favouring folded conformations, and by structuring intermediates.

Examples in the literature showed that similar mechanisms may underlie the observed gain of function of destabilised variants of human proteins by chemical chaperones. That is for example the case of the cytosolic cystathionine β-synthase (CBS). The deficient activity of CBS, a homotetrameric enzyme involved in sulphur metabolism, leads to the metabolic disease classic homocystinuria (OMIM 236200). The expression of mutant forms of CBS in a yeast expression model, in the presence of a range of chemical chaperones (TMAO, glycerol, sorbitol, proline and DMSO) induced an increase in activity and in the tetrameric assembly of the protein [33]. However, the rescue of enzyme activity was only detected when the mutant variants were expressed in the presence of the studied compounds, and not when these were added to the purified mutant protein. Interestingly, a synergistic effect resulting from the combination of these compounds was observed. This led to the suggestion that in this case, the studied osmolytes exert their effect by favouring productive folding pathways, possibly by minimising intermediate destabilised conformations, as observed for barstar.

Effect on Oligomerization

If the mutant protein is a component of a heteromeric complex, then the stabilisation of an intermediate state may also induce a rescue of the assembly of the complex. This was observed for the mitochondrial branched-chain β-ketoacid dehydrogenase (BCKD) complex. Using an *in vitro* system, Song and colleagues showed that TMAO corrected the assembly defects found in Maple Syrup Urine Disease (MSUD) type IA, which are caused by mutations in the heterotetramer $E1\alpha_2\beta_2$ component of the BCDK complex [34]. When mutant forms of E1α subunit are present, inactive heterodimers are formed which prevent the heterotetrameric assembly of the complex $\alpha_2\beta_2$. In the presence of TMAO, inactive heterodimers are converted into active heterotetramers. It has been suggested that TMAO destabilises the folded mutant heterodimers through hydration, thus increasing its Gibbs free energy, even after mutant E1 has been folded and misassembled. This unstable state will induce the folding of the unfolded protein into a native conformation that will then correctly oligomerize.

Stabilisation of the Native Conformation by Ligands, Inhibitors and Cofactors

The stabilisation of the native state by specific small molecules which prevent transitions to pathological conformations, is one of most promising therapeutic applications of chemical chaperones. Indeed, the use of compounds that act over a particular protein, such as inhibitors, ligands or cofactors, assures the required pharmacological specificity [16,18,29].

In the last years, transthyretin (TTR) has been one of the most explored target protein for stabilisation by low molecular weight compounds. TTR is a 55-kDa homotetrameric protein found in plasma and cerebrospinal fluid that transports thyroid hormones and the retinol/retinal-binding protein complex. TTR mutations are involved in FAP, the large majority of which destabilise the functional tetrameric state. This results in partial denaturation of the monomer, with subsequent assembly into amyloid and aggregate forms. The native ligand thyroxine presents the capacity to inhibit fibril formation by stabilizing the TTR tetramers against dissociation, thereby preventing the subsequent conformational changes required for amyloid fibril formation [35,36]. A series of TTR inhibitors have been developed, aimed at slowing the initial misfolding event of tetramer dissociation, that function by binding to the two thyroxine sites in the dimmer-dimer interface [37]. Also, many TTR ligands (such as diflunisal, diclofenac, flufenamic acid) and amyloid disrupters (such as 4'-iodo-4'-deoxydoxorubicin and tetracyclines) have been suggested as possible therapeutic agents in TTR amyloidoses [38]. For example, tetracyclines have also been shown to disrupt TTR amyloid fibrils *in vitro*, producing noncytotoxic species [39], a result which has been subsequently corroborated *in vivo* using a FAP transgenic mice model [40]. A recent study has investigated the ability of several TTR fibrillogenesis inhibitors to inhibit TTR-L55P aggregate formation. A cellular system that produces TTR intermediates/aggregates in the medium was used to perform the screening, and the compounds 2-[(3,5-dichlorophenyl) amino] benzoic acid, benzoxazole, 4-(3,5-difluorophenyl) benzoic acid and tri-iodophenol were found to be the most effective inhibitors, as compared with the reference iodiflunisal, previously shown by *ex vivo* and *in vitro* procedures to stabilise TTR and inhibit fibrillogenesis [41].

Along a different line, the studies of Chiti and colleagues have also suggested that stabilisation of the native structure by specific binding of a ligand may be an effective strategy against protein deposition disorders that result from proteins which are initially folded. Their assumption is based on studies on acylphosphatases (AcP), which may undergo conversion to amyloid-type aggregates without prior unfolding. In these proteins, interaction of the native state with inorganic phosphate, a competitive inhibitor of AcPs, inhibits the amyloid conversion step [42].

Rescue of Folding and Trafficking Defects

Proteins whose functional localization is the cytoplasmatic membrane, the extracellular milieu or the lysosome, are trafficked through the Golgi up to their destination after being synthesized and folded at the ER. During this process,

misfolded proteins will be recognized by the protein quality control (PQC) system and targeted for degradation. This yields a decreased protein level at the final localization, and eventually the onset of a pathologic condition. The ER folding environment can be manipulated using chemical chaperones that will prevent the degradation of mutant proteins by the ER-associated degradation (ERAD). These will improve folding and trafficking and will restore the functional deficiency at the final destination, providing that the trafficked protein is stable in its destination milieu.

Among traffick-defective proteins, lysossomal mutant enzymes responsible for the development of a large number of severe human diseases such as Gaucher's, Fabry´s and Pompe's disease, Table (**1**), they have been major targets for chemical chaperoning therapies. Gaucher's disease is caused by mutations in the gene codifying for the lysossomal enzyme glucocerebrosidase (GC). Several misfolded mutant GC forms accumulate in the ER, with subsequent targeting for ERAD. GC synthetic inhibitors, namely N-(n-nonyl)deoxynojirimycin (NN-DNJ) and N-(n-butyl)deoxynojirimycin (NB-DNJ), have been used to correct the folding and ER accumulation of GC variants, restoring the proper traffic to the lysosome [23,24]. *In vitro* these low molecular weight compounds, which bind to the GC active site, increased the thermal stability of several mutant forms resulting in a more stable protein [24]. Natural inhibitors are also able to restore the localization of lysossomal proteins, as in the case of the α-galactosidase A (GLA). Mutant forms of GLA are responsible for Fabry's disease, an X-linked inborn error of glycosphingolipid catabolism. Misfolded forms of GLA are stabilised by its reversible natural competitive inhibitor galactose [43] and by the synthetic iminosugar competitive inhibitor 1-deoxy-galactonojirimycin (DGJ) [44].

Defective membrane proteins, involved in the development of pathological conditions have also been targets of stabilisation by chemical chaperones. In this particular case, one of the most studied proteins is the epithelial cAMP-regulated Cl^- channel, the cystic fibrosis transmembrane conductance regulator (CFTR) [45]. Defective function of CFTR causes Cystic fibrosis a common genetic disease in the Caucasian population. Several mutant forms of CFTR, and particularly the ΔF508, are trafficking-impaired mutations since the synthesized protein is retained in the ER and degraded, rather than trafficked to the cell surface. An increase in the expression of mutant forms of CFTR on the epithelium surface was achieved using natural osmolytes [46,47] (glycerol, betaine, taurine and TMAO among others), deuterated water [14], the isoflavone genistein [48] and natural products [49] including phytoflavonoids. As a result of high throughput drug screening studies for CFTR maturation correctors [50,51], effective synthetic ligands were identified. Furhter studies have shown that these interact at different sites of the protein, and have an additive effect on the maturation of ΔF508 CFTR [52]. Likewise, the incorrect localization of the transmembrane Cu^{2+} transporting P-type ATPase (MNK), a protein located in the trans-Golgi network (TGN) where it transports copper to the copper-/dependent enzymes synthesized within secretory compartments [53] was rescued using its metal ion ligand (copper). Mutations in MNK are responsible for Menkes disease, an X-linked copper deficiency disorder. Using a mammalian cell model it was demonstrated that copper restored the normal perinuclear localization of the G1019D mutant form of MNK and increased the amount of the normal sized product (properly glycosylated). It has been suggested that the binding of copper to the MNK copper binding sites may serve to promote a conformation that is able to bypass the ER quality control mechanisms [53].

Another example is given by α1-antitripsin (α1-AT), an extracellular protein synthesized in the ER. In α1-antitripsin deficiency a mutant α1-AT protein (α1-ATZ) is retained in the ER resulting in a reduction of plasmatic α1-AT levels. It has been postulated that the pathogenic condition is caused by the accumulation of the misfolded protein in the ER (ER stress). Burrows and colleagues [54] demonstrated, in a human fibroblast cell line, that glycerol and 4-PBA, but not TMAO, D_2O and betaine, were able to induce an increment on the extracellular content of α1-ATZ by a mechanism involving an increase in the translocation from the ER into the rest of the secretory pathway rather than an effect on ER degradation of α1-ATZ.

Modulation of Protein Quality Control System and Unfolded Protein Response (UPR) System

When ER quality control systems are overwhelmed (ER stress), cells use the Unfolded Protein Response (UPR) system to assist in managing the protein overload in the ER [55]. The UPR enhances the levels of molecular chaperones involved in protein folding and degradation and reduces the rate of protein synthesis. This system is mainly regulated by three signal transducers namely the inositol-requiring 1 (IRE-1), the protein kinase-like ER kinase (PERK) and the activating transcription factor 6 (ATF6). These attenuate overall translation and activate the transcription of the genes involved in the UPR, namely specific molecular chaperones. It is now well established that several low molecular weight compounds are able to indirectly interact with the UPR system by modulating the expression of the genes involved in this pathway.

This is the case of 4-PB and tauroursodeoxycholic acid (TUDAC). These compounds effectively reduced the ER stress levels on leptin-deficient (ob/ob) mice [56], a model of severe obesity and type 2 diabetes. usually associated with the development of insulin resistance. This was achieved by reducing PERK and IRE-1 phosphorylation, thus resulting in an improved insulin signalling in liver and adipose tissues. An identical action was also recently identified in the hereditary hemochromatosis (HH) protein [57]. In this case, misfolding of the mutant protein HFE C282Y at the ER results in its targeting for degradation. Whereas TUDCA increased stability of the mutant protein therefore reducing ER stress, 4-PB prevented its degradation, facilitating the degradation of misfolded HFE.

Stabilisation of Aggregation Prone Proteins

The pathological processes in polyglutamine disorders, such as Huntington's disease (HD) and Machado-Joseph's disease (MJD), have been proposed to be related with the intracellular accumulation of aggregates of polyglutamine containing proteins, namely huntingtin (in HD) and ataxin-3

(in MJD). Based on an *in vitro* model system using myoglobin (Mb) as a host protein for glutamine repeats of various lengths (Mb-$Gln_{12,28,35\ and\ 50}$), Tanaka and colleagues [58] were able to demonstrate that various disaccharides, and particularly trehalose, had the potential to minimize the aggregation propensity and increase the stability of the partially unfolded Mg-$Gln_{repeats}$. Furthermore, in a transgenic mouse model of HD, trehalose was able to alleviate the polyglutamine-mediated pathology. In Alzheimer's disease, in which amyloid plaques are fibrillar structures of assembled amyloid-β peptide, amyloid formation was modulated by TMAO, glycerol [59] and several carbohydrates [60]. Glycerol and TMAO promoted the stabilisation of the lower energy native state conformer, therefore reducing the levels of unfolded protein that is required for the amyloidogenic pathway. Sugar molecules at low concentrations are hypothesised to favour H-bonding interactions that minimize cytotoxic conformations. However, the possibility that the some of the effects of sugars observed in *in vivo* models results from an up-regulation of the protein chaperones, and not from direct protein stabilisation, remains to be considered.

Nucleation Effects in Intrinsically Unstructured Proteins

Another important action of chemical chaperones relates to the fact that some compounds may promote gain of structure in intrinsically unstructured proteins [61,62], therefore regulating a particular cellular or biochemical process. The tumour suppressor protein p53 is an intrinsically unstructured protein and it has been suggested that the lack of a rigid structure combined with a low overall stability may be essential features for its physiological action, regulation and turnover [63]. Several osmolytes were shown to have a chaperoning effect over p53 [19]. In the presence of TMAO, glycerol or D_2O, cell lines expressing temperature sensitive mutants of p53 incubated at non-permissive temperatures promote restoration of the phenotype. Since the phenotype remained after removal of the chemicals, it has been suggested that these induced a wild-type like protein conformation which remained in a physiologically compatible conformation [64].

5. A BRIEF CASE STUDY: RESCUE OF FOLDING DEFECTS IN CLINICAL PAH VARIANTS

This section will provide a brief overview concerning the effect of chemical chaperones on the rescue of protein folding defects observed in PAH in the context of PKU mutations. Phenylketonuria (PKU; OMIM 261600) is considered a paradigm of genetic disorders, and has a high potential for the development of therapeutic approaches using chemical chaperones. PKU is the most frequent inborn error of amino acid metabolism, presenting an average frequency of 1:10000 among Caucasians. It is caused by mutations in the gene coding for the cytosolic enzyme phenylalanine hydroxylase (PAH; EC 1.14.16.1.), a homotetrameric protein that requires dioxygen and the cofactor (6R)-L-erythro-5,6,7,8-tetrahydrobiopterin (BH_4) to hydroxylate L-phenylalanine (L-Phe) to L-tyrosine (L-Tyr). Presently over 500 different mutations have been identified [65,66] thus contributing to a high level of phenotypic heterogeneity, which can range from severe (classic PKU) to benign clinical presentations (HPA non-PKU). In order to prevent the psychomotor delay due to high circulating levels of L-Phe, PKU patients follow a dietary restriction of L-Phe intake throughout their lives. While during the first years of life the dietetic treatment is effective in maintaining L-Phe therapeutic levels, adolescents and young adults present a poor diet compliance, with the resulting neurological impairment. Moreover, a strict control of blood L-Phe levels in pregnant PKU women is needed in order to prevent congenital malformations of the foetus (maternal PKU). Therefore, alternative treatments for PKU have been searched for.

The *in vitro* analysis of heterologous expressed PAH missense mutations has shown that the majority of the characterized enzymes result in misfolding of PAH, increased protein degradation turnover and a loss of enzymatic function. Recently, the energetic impact of 318 PKU-associated missense mutations on PAH native-state stability was computationally predicted, suggesting that the decrease in PAH stability is the main molecular pathogenic mechanism in PKU, and the determinant for phenotypic outcome [67]. As a result, PKU may be classified as a conformational disorder.

PAH is a homotetrameric enzyme, containing a non-heme mononuclear iron site. PAH consists of an N-terminal regulatory domain, a catalytic domain and a C-terminal domain, responsible for tetramerization. This enzyme is regulated by three main mechanisms, namely substrate activation, pterin cofactor inhibition and phosphorylation of a single serine (Ser16) residue. The natural cofactor BH_4 exerts a negative regulatory effect inducing a low-activity conformational state, blocking L-Phe binding site and phosphorylation of Ser16 [68].

In 1999, Kure and co-workers [69] reported several PAH deficient patients presenting a reduction in blood serum L-Phe levels after oral administration of BH_4. This suggested a chaperone-like effect of the pterin, which could stabilise the misfolded mutant protein. Following this report, several studies were carried out, establishing the molecular mechanism underlying the observed effect. Using transgenic mice, with complete or partial deficiency in BH_4 biosynthesis [70], and transfected human hepatoma cells [71], an effect on transcriptional regulation or on mRNA stabilisation by the addition of the cofactor was excluded. Using an *in vitro* cell free expression system it was shown that the mutant enzymes, produced in the presence of BH_4 presented a decreased rate of protein degradation and inactivation [72,73]. This effect was postulated to be a result of a chaperone-like effect of the cofactor, lowering the PAH degradation rate. However, since a similar response was obtained when the PAH proteins were produced in the presence of catalase and superoxide dismutase, a role of the cofactor in the prevention of the chemical inactivation of PAH enzymes by oxidation could not be excluded. It was hypothesized that binding of BH_4 at saturating concentrations might prevent peroxide formation, which inactivates PAH, and protects the right configuration of the active site. Clearly the molecular mechanism underlying the observed decrease in the L-Phe circulating levels after oral administration of BH_4 must be more complex than initially predicted. Further, the response to oral intake of BH_4 in PKU patients presenting different phenotypes showed that it was dependent of the patient's

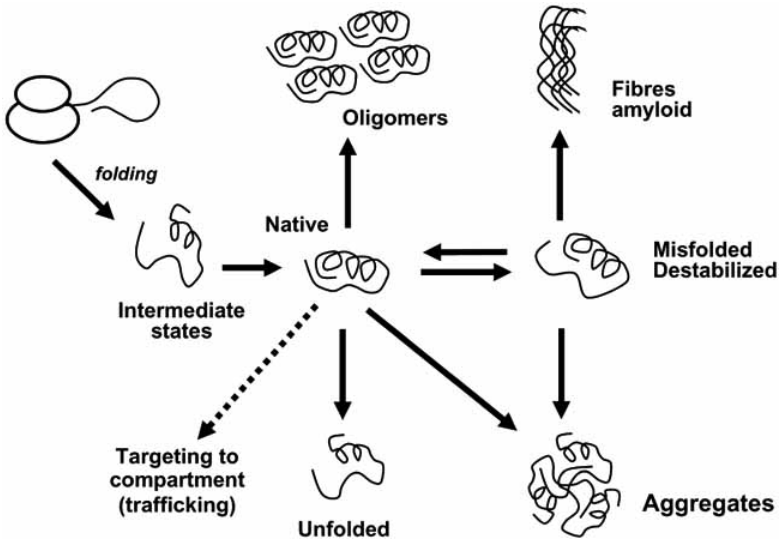

Fig. (5). Glycerol improves PAH structure and conformation of the PKU-related R261Q variant.

Heterologous expression of the human PAH R261Q variant in the presence of a physiologically compatible concentration of glycerol (5 mM) substantially improved the oligomerization of PAH into the functional tetrameric form as shown by size exclusion chromatography (Panel A) and rescued PAH folding to near wild-type conformation, as shown by far-UV CD (Panel B) (Leandro and Gomes, personal communication).

genotype, being more prevalent in patients with mild hyperphenylalaninemia and mild PKU [74]. Nevertheless, and although the molecular mechanism remains unknown, the demonstration that BH_4 responsiveness is more frequent than what was previously assumed led to the approval of its introduction in pharmacological preparations for the treatment of PKU.

Natural osmolytes have also been shown to stabilise mutant forms of PAH. The addition of glycerol to prokaryotic cells expressing mutant forms of PAH induced the synthesis of the recombinant proteins at higher levels and lead to an increase in its catalytic activity [75]. This effect was also obtained after addition to the culture medium of TMAO (5 mM) and taurin (10 mM) but not with 4-PB. The presence of osmolytes also reduced the aggregated forms, and resulted in an increase of the active tetrameric forms. Further characterization of the tetramers using far-UV circular dichroism revealed the presence of more structured forms resembling a native-like state, Fig. (**5**). The observed increase in the enzymatic activity of PAH mutants obtained in this study (from 20% to near 80% residual activity) by natural chemical chaperones [75] is a challenge to the identification of additional low molecular weight compounds which could be used to restore the enzyme activity of mutant PAH proteins.

6. OUTLOOK AND PROSPECTIVE

It is nowadays clear that chemical chaperoning can be an effective approach to overcome protein folding and trafficking defects. In this review we have shown that the proof of principle of the action of chemical chaperoning has been established on solid grounds for a large number of proteins, many of which are involved in human diseases. The diversity of studies which we have overviewed illustrate that small chemical compounds have rather diverse modes of action, from a direct effect on protein conformations and folding pathways, to a modulation of cellular protein quality control systems. As a result, many of these compounds are being currently used with therapeutic purposes in clinical trials. The use of synthetic inhibitors to treat Gaucher´s disease and of curcumin, xanthines and flavonoids, such as genistein, in clinical pilot studies in cystic fibrosis patients are concrete examples of the potential pharmacological applications of chemical chaperones. For Gaucher´s disease, one such example is isofagomine, which is currently being used in a clinical trial [76].

However, the broad application of these compounds in a therapeutic perspective has still numerous challenges to meet. Issues relating to dosage, toxicity, crossing of blood-brain barrier, and delivery to particular cell types or cellular compartments are some examples of the problems that will have to be addressed when attempting to translate this research to treat patients. The perspective is that the pursuit of these studies will firmly contribute to a better molecular and cellular understanding of chemical chaperoning which ultimately may have an impact in the discovery of new therapeutic targets and in the design of candidate drugs for the treatment of conformational disorders.

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