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REVIEW

Phenylketonuria as a model for protein misfolding diseases and for the development of next generation orphan drugs for patients with inborn errors of metabolism

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Abstract The lecture dedicated to Professor Horst Bickel describes the advances, successes, and opportunities concerning the understanding of the biochemical and molecular basis of phenylketonuria and the innovative treatment strategies introduced for these patients during the last 60 years. These concepts were transferred to other inborn errors of metabolism and led to significant reduction in morbidity and to an improvement in quality of life. Important milestones were the successful development of a low-phenylalanine diet for phenylketonuria patients, the recognition of tetrahydrobiopterin as an option to treat these individuals pharmacologically, and finally market approval of this drug. The work related to the discovery of a pharmacological treatment led metabolic researchers and pediatricians to new insights into the molecular processes linked to mutations in the phenylalanine hydroxylase gene at the cellular and structural level. Again, phenylketonuria became a prototype disorder for a previously underestimated but now rapidly expanding group of diseases: protein misfolding disorders with loss of function. Due to potential general biological mechanisms underlying these disorders, the door may soon open to a systematic development of a new class of pharmaceutical products. These pharmacological chaperones are likely to correct misfolding of proteins involved in numerous genetic and nongenetic diseases.

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Abbreviations

PKU	Phenylketonuria
PAH	Phenylalanine hydroxylase
BH_4	Tetrahydrobiopterin
ER	Endoplasmic reticulum
ERAD	Endoplasmic reticulum associated degradation

Prof. Dr. Dr. h.c. Horst Bickel, 1918–2000

From an untreatable to a treatable disorder: the development of a low-phenylalanine diet for phenylketonuria

Phenylketonuria (PKU; MIM 261600) is caused by a deficiency of hepatic phenylalanine-4-hydroxylase (PAH; EC 1.14.16.1) due to mutations in the *phenylalanine hydroxylase* (*PAH*) gene. In the early 1950s, Dr. Horst Bickel successfully developed a low-phenylalanine diet for patients suffering from what was considered an untreatable disorder. He had diagnosed PKU in a 17-month-old girl, Sheila, who had severe mental retardation. The persistent

and determined mother insisted on receiving treatment for her daughter and spurred Dr. Bickel to action (Koch 1997). He used a new technology to remove single amino acids from a protein hydrolysate by filtration through activated charcoal to produce the first phenylalanine-free formula. Casein hydrolysate was widely available because it had been produced for people suffering from severe malnutrition following the Second World War. Indeed, under treatment the girl improved, her hair grew darker, she no longer cried continuously, and she stopped banging her head. Moreover, she learned to crawl, to stand, and to climb on chairs. A first paradigm change was achieved: phenylketonuria was the first treatable inherited disorder (Bickel et al. 1953, 1954).

From dietary to pharmacological treatment: the development of a cofactor therapy for phenylketonuria

At the end of the last century, metabolic pediatricians recognized that some phenylketonuria patients may benefit from pharmacological doses of the natural cofactor tetrahydrobiopterin (BH_4) of the deficient enzyme PAH. The substance had been used for 20 years to perform a BH_4 loading test in order to distinguish patients with an apoenzyme deficiency from those very rare patients with a genetic cofactor deficiency necessitating BH_4 substitution. In 1999, Kure et al. published a landmark paper describing four patients with phenylalanine hydroxylase deficiency without any evidence of a defect in synthesis or regeneration of the cofactor. They responded to a BH_4 load with a decrease in serum phenylalanine concentrations (Kure et al. 1999). Several other groups subsequently confirmed this observation (Lässker et al. 2002; Lindner et al. 2003; Shintaku et al. 2004; Spaapen et al. 2001; Steinfeld et al. 2002, 2004; Trefz et al. 2001; Weglage et al. 2002). In a systematic clinical study involving 38 patients, the majority of patients with mild phenotypes benefited from cofactor treatment. BH_4 reduced blood phenylalanine concentrations and increased dietary phenylalanine tolerance by increasing in vivo PAH activity (Muntau et al. 2002). In 2007 (FDA) and 2008 (EMEA), sapropterin dihydrochloride, the synthetic form of BH_4, was approved as an orphan drug to treat patients with BH_4-responsive PAH deficiency (Feillet et al. 2008; Lee et al. 2008; Levy et al. 2007; Trefz et al. 2009). This achievement marked a second paradigm change, the partial or full replacement of an effective but burdensome diet with an oral pharmacological treatment. About 60% of all PKU patients are estimated to benefit from this new therapy. Notably, patients with a mild to moderate clinical phenotype due to a genotype associated with some residual enzyme activity are more likely to respond to cofactor treatment than those with classic PKU carrying nonsense mutations (Bernegger and Blau 2002; Hennermann et al. 2005; Muntau et al. 2002; Wang et al. 2007; Zurflüh et al. 2008).

Phenylketonuria: from a biochemical enzyme deficiency to a protein misfolding disorder with loss of function

At the time of approval of sapropterin dihydrochloride to treat phenylalanine hydroxylase deficiency, the mode of action of the new drug was not well understood. Some proposed that the restoration of enzyme function with pharmacological doses of BH_4 occurs through correction of PAH misfolding. The view of protein misfolding being the molecular mechanism in PAH deficiency was supported by previous studies demonstrating disturbed oligomerization, decreased stability, and accelerated degradation of variant PAH proteins (Bjørgo et al. 1998; Gjetting et al. 2001; Kim et al. 2006; Pey et al. 2003, 2007; Waters 2003; Waters et al. 2000, 2001). To further clarify the molecular basis for functional impairment in PAH deficiency, we investigated the impact of ten *PAH* mutations identified in BH_4-responsive patients. Residual enzyme activity was generally high, but allostery was disturbed in almost all cases and suggested altered protein conformation. Thus, mutations in the *PAH* gene do not predominantly affect the catalytic function of the protein but rather impair molecular motions needed for regulatory processes such as activation or inhibition, substrate and cofactor binding, and to promote changes of the oligomeric state. This was confirmed by demonstrating reduced proteolytic stability, impaired tetramer assembly or aggregation, increased hydrophobicity, and accelerated thermal unfolding with particular impact on the regulatory domain of the enzyme (Gersting et al. 2008). Figure 1 summarizes the molecular processes leading to loss of function of the PAH protein. Single amino acid substitutions due to missense mutations can induce protein misfolding, aggregation, and early degradation. All processes are likely to be mutually dependent. Thus, distinguishing the effects of the efficiency of PAH protein folding into the native state from the effects on the rate of misfolding is difficult. The combined action of all disturbances leads to loss of functional PAH resulting in impairment of the phenylalanine hydroxylating system.

BH_4: from a natural enzyme cofactor to a pharmacological chaperone

After the discovery of the cofactor treatment for PAH deficiency, several hypotheses to explain the BH_4 effect were proposed. Considering PKU is a protein misfolding disease with loss of function, the possibility that BH_4 exerted its action as a pharmacological chaperone was examined. A pharmacological chaperone is a small molecule that rescues

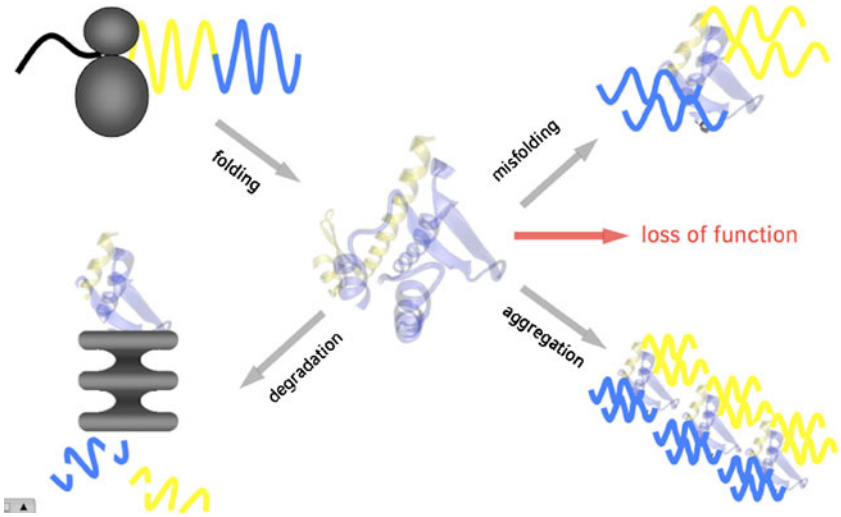

Fig. 1 Schematic view of the molecular pathophysiology of PAH deficiency. *PAH* mutations can lead to protein misfolding, aggregation, and early degradation and thus to a loss of functional PAH. All processes are mutually dependent and contribute to loss of function

protein function by improving protein folding and by stabilizing the protein structure. In vitro studies on recombinantly expressed PAH pointed to stabilization of the misfolded protein by BH_4 against denaturation and degradation (Aguado et al. 2006; Erlandsen et al. 2004; Pérez et al. 2005; Pey et al. 2004). Pharmacological doses of the cofactor also stabilized wild-type PAH protein levels in mouse liver (Scavelli et al. 2005; Thöny et al. 2004). At the time of BH_4 approval, an animal model exhibiting the particular phenotype of BH_4-responsive PAH deficiency was unavailable. This prevented the elucidation of the mode of action underlying pharmacological chaperone therapy in vivo. We recently showed that the Pah^{enu1} mouse is a model for BH_4-responsive PAH deficiency (Gersting et al. 2010). Pah^{enu1} was generated by germline mutagenesis, carries a mutation leading to a V106A amino acid substitution in the regulatory domain, and shows a mild hyperphenylalaninemia phenotype (McDonald et al. 1990; Scavelli et al. 2005; Shedlovsky et al. 1993; Thöny et al. 2004). We demonstrated that loss of function in this animal results from loss of PAH, a consequence of misfolding, aggregation, and accelerated degradation of the enzyme. BH_4 attenuates this triad by conformational stabilization augmenting the effective PAH concentration, which is the amount of functional PAH (Fig. 2). This leads to rescue of the biochemical phenotype and enzyme function in vivo.

Although general rules defining BH_4-responsive genotypes have not been established, it is evident that the degree of misfolding is limited to an extent that allows for some residual specific activity to provide reasonable enzyme function of the stabilized protein. Moreover, combined in vitro and in vivo analyses demonstrated a selective BH_4 pharmaceutical action confined to the pathological metabolic state reflecting the physiological inhibitory action of

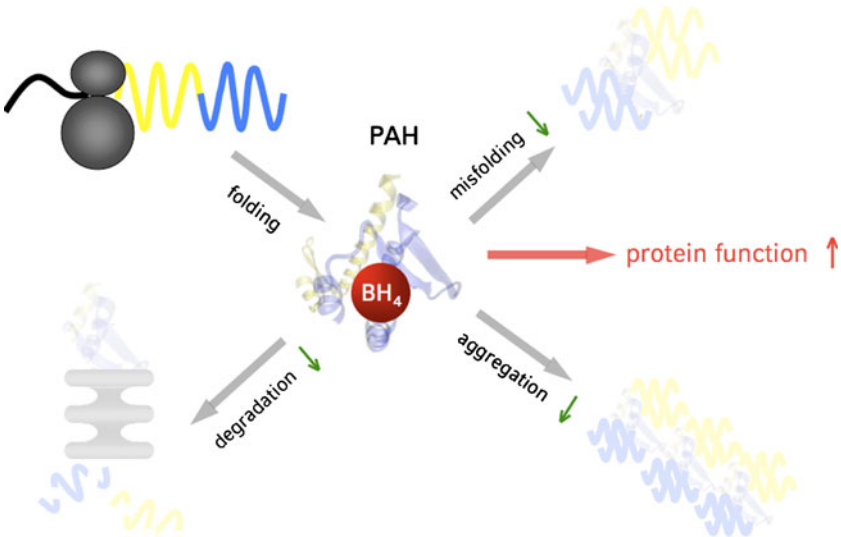

Fig. 2 Molecular mode of action of BH_4. At the protein level, BH_4 prevents misfolding, aggregation, and degradation, and thus induces an increase in the effective PAH concentration resulting in rescue of its function

Table 1 Pharmacological chaperone treatments for lysosomal storage diseases

Disease	Enzyme deficiency	Pharmacological chaperones	Literature
Fabry disease	GLA	DGJ	Fan et al. 1999
Gaucher disease	GBA	IFG, DNJ	Yu et al. 2007
G_{M1} gangliosidosis	GLB1	NOEV	Matsuda et al. 2003
G_{M2} gangliosidosis	HEXA	Pyrimethamine	Maegawa et al. 2007
Pompe disease	GAA	DNJ	Parenti et al. 2007
MPS IIIB	NAGLU	2AcDNJ and 6AcCAS	Ficko-Blean et al. 2008

GLA α-Galactosidase A, *GBA* acid beta-glucosidase, *GLB1* beta-galactosidase 1, *HEXA* hexosaminidase A, *GAA* α-1, 4-glucosidase, *NAGLU* α-*N*-acetylglucosaminidase

the cofactor BH_4. This is of particular importance since it prevents undue removal of the essential amino acid phenylalanine and thus protects the patient from overtreatment (Gersting et al. 2010). These data show, after market approval, that the drug BH_4 is indeed the first pharmacological chaperone. Future developments will focus on the identification and the design of BH_4 derivatives or new molecules that also stabilize the PAH protein but show more favorable pharmaceutical properties. A high-throughput ligand screen of over 1,000 pharmacological agents already revealed two alternative compounds that stabilize the functional tetrameric conformation of the PAH protein (Pey et al. 2008).

Protein misfolding and pharmacological chaperone therapy for genetic inborn errors of metabolism—an emerging field

A number of inborn errors of metabolism have now been recognized to be associated with protein misfolding and loss of function. They can therefore be considered excellent candidates for pharmacological chaperone therapy (Mu et al. 2008).

Lysosomal storage diseases

Protein misfolding with loss of function was shown for some lysosomal enzymes linked to lysosomal storage diseases (Fabry disease, Gaucher disease, gangliosidosis G_{M1} and G_{M2}, Pompe disease, and mucopolysaccharidosis IIIB; Fan et al. 1999; Futerman and van Meer 2004; Sawkar et al. 2002; Schmitz et al. 2005). These proteins are synthesized in the endoplasmic reticulum. Misfolded proteins are recognized by the ER-related quality control system for newly synthesized proteins, they are retrotranslocated into the cytosol and degraded by the ER-associated degradation (ERAD) machinery (Brodsky 2007; Cohen and Kelly 2003; Schröder and Kaufman 2005; Ulloa-Aguirre et al. 2004; Vembar and Brodsky 2008). Thus, misfolded lysosomal enzymes do not reach the lysosome, their target destination, to exert their function. Small molecules acting as pharmacological chaperones have been identified, and treatment with some is being clinically investigated. Lysosomal storage diseases associated with protein misfolding and potential stabilizing substances are summarized in Table 1.

Fatty acid oxidation defects

In many mitochondrial inherited metabolic disorders, missense mutations affect the folding propensity of the protein and the stability of the native protein conformation; this has been extensively investigated for acyl-CoA dehydrogenases (Bross et al. 1995, 1999; Gregersen et al. 2006; Gregersen and Olsen 2010; O'Reilly et al. 2005; Saijo et al. 1994). A study analyzing the impact of ten *ACADM* mutations identified by expanded newborn screening and associated with mild to asymptomatic phenotypes revealed severe effects on conformation, stability, and enzyme kinetics of the corresponding recombinant medium chain acyl-CoA dehydrogenase proteins. Partial to total rescue of aggregation by co-overexpression of GroESL confirmed

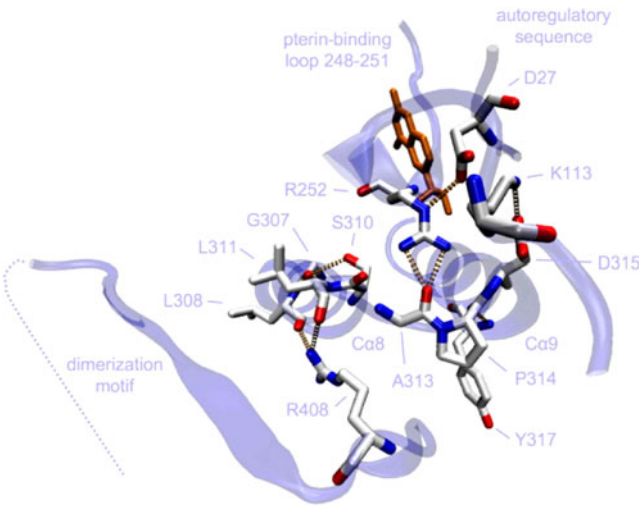

Fig. 3 A structural network comprising side chains in different domains of the PAH monomer. Side chains located in different functional parts of the protein (autoregulatory sequence, pterin-binding loop, dimerization motif) are connected via hydrogen bonds or through backbone structures. Ranging from D27 to R408, they virtually span the whole primary sequence of PAH (Gersting et al. 2008)

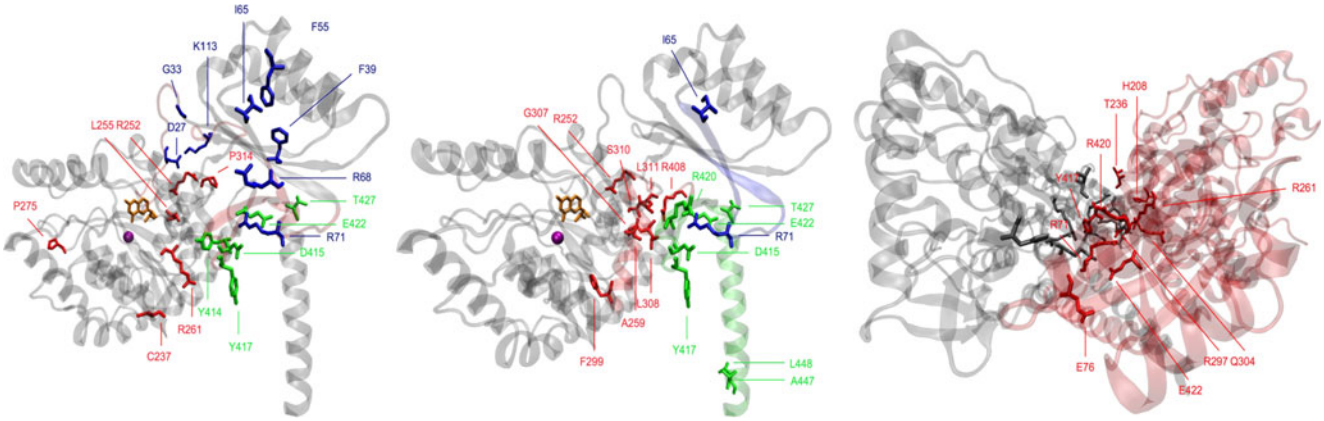

Fig. 4 Amino acid residues involved in structural and functional side chain networks within the PAH monomer/dimer. *Left* Activation, *middle* oligomerization, *right* monomer/monomer interface. Residues of the three functional domains are highlighted (*blue* regulatory domain, *red* catalytic domain, *green* oligomerization domain). Residues of the regulatory domain would be expected to be particularly part of the activation process. Instead, residues from all three domains are involved (Gersting et al. 2008)

protein misfolding. In most variants, thermal unfolding and thermal inactivation were accelerated, and proteolytic stability was decreased (Maier et al. 2009). In short-chain acyl-CoA dehydrogenase deficiency, aggregation seems to play an important role in pathogenesis (Pedersen et al. 2003).

Disorders of homocysteine metabolism

The first case of human S-adenosylhomocysteine (AdoHcy) hydrolase deficiency was described a few years ago (Baric et al. 2004). The disorder leads to slow psychomotor development, severe muscular hypotonia with elevated serum creatine kinase and transaminases, and white matter atrophy in the CNS. Further molecular studies revealed that one variant derived from the mutation Y143C leads to early thermal unfolding and to increased thermal inactivation classifying AdoHcy hydrolase deficiency as a protein misfolding disease with loss of function (Beluzic et al. 2006).

The structural basis of protein misfolding and pharmacological chaperone treatment

Genetic mutations reduce protein stability, accelerate protein degradation, and lead to reduced effective concentration of the functional enzyme in the cell. These data provided indirect experimental evidence for protein misfolding. However, analyses of the structural basis of molecular mechanisms underlying protein misfolding are required to gain direct insight into the molecular and structural events linked to protein misfolding. In a further stage, the structural mechanisms of pharmacological chaperone treatment can be elucidated. A prerequisite for these studies is a 3D-structural model of the

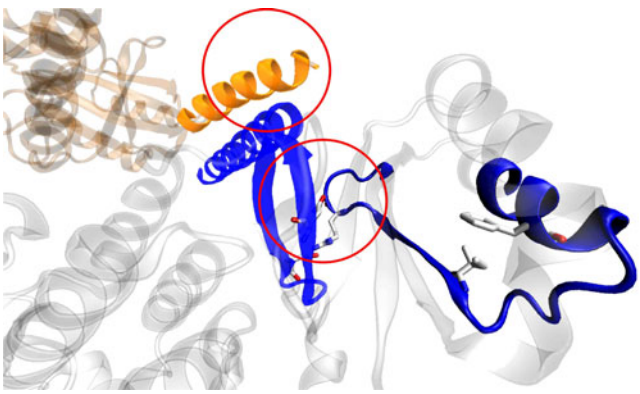

Fig. 5 The N-terminal amino acid replacement I65S affects remote parts of the PAH protein. It dislocates the alpha-helical tetramerization motif and by this disrupts the coiled-coil interaction essential for correct tetramer assembly at the C-terminus of the protein

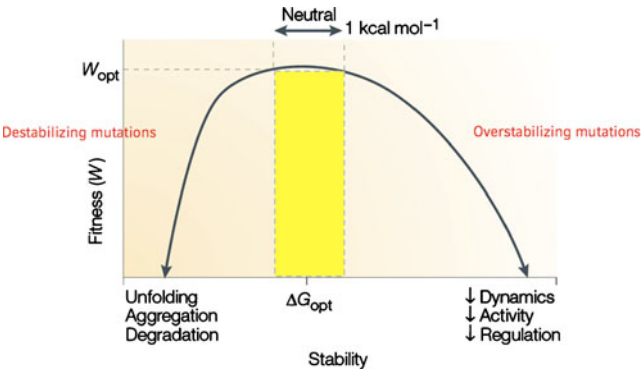

Fig. 6 The relationship between stability (ΔG) and fitness (W). The range of optimized thermodynamic properties (ΔG_{opt}) for best fitness of a protein is narrow. Destabilizing mutations lead to a decrease on the left side with protein unfolding, aggregation, and degradation. On the right hand side, the decrease in fitness results from a reduction in dynamics, activity, and regulation owing to overstabilization (DePristo et al. 2005)

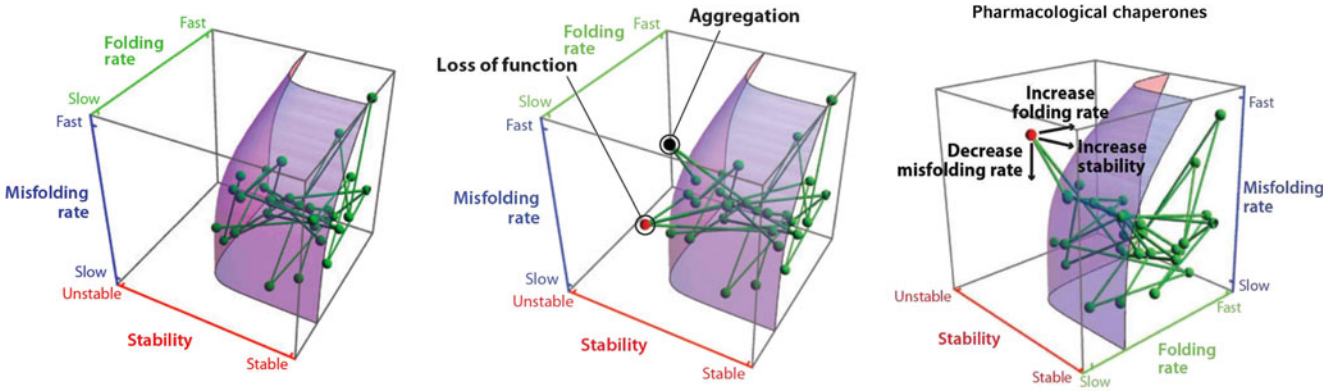

Fig. 7 A model for proteostasis and the effect of mutations and pharmacological chaperones. Nodes represent a protein's folding energetics. The *purple surface* represents the proteostasis boundary. *Left* In the healthy cell, all nodes are within the proteostasis boundary. *Middle* Mutations can alter the folding energetics of the proteins leading to loss of function or to aggregation and making their nodes fall outside the proteostasis boundary. *Right* Pharmacological chaperones rescue protein function by decreasing the misfolding rate and by increasing protein stability (Powers et al. 2009)

target protein. The first and so far only publication describing the structural consequences of pharmacological chaperone binding to its target protein refers to Gaucher disease (Lieberman et al. 2007).

For phenylketonuria, a composite model of PAH is commonly used for 3D structural analysis, since the crystal structure of full-length tetrameric PAH is unavailable (Andersen et al. 2001). However, molecular modeling must rely on robust data to generate meaningful predictions in terms of mutational (Pey et al. 2007) or drug effects (Allen et al. 2007). These will enable further studies to directly analyze the conformational rearrangements of the complete PAH tetramer upon binding of the substrate and the pharmacological chaperone BH_4.

The concept of protein misfolding with loss of function: a general molecular consequence of missense mutations?

Recent studies identified that the local conformation of single amino acid residues at one site can control the structure of remote parts of the protein (Allen et al. 2007; Daily and Gray 2007; Daily et al. 2008; Tekpinar and Zheng 2010). Concerning PAH, it was shown how amino acid side chains can be involved in different structural and operational networks shaping form and function of the protein (Fig. 3) (Andersen et al. 2003; Stokka et al. 2004; Thórólfsson et al. 2003). Amino acid side chains network through direct interactions with other side chains via hydrogen bonds, phi stacking, or van der Waals forces. In addition, backbone regions involved in conformational rearrangements contribute to these networks, so that clusters spanning more than one domain are formed (Fig. 4). Thus, single amino acid replacements at one site affect the function and stability of other regions or domains of the protein. This phenomenon can be explained by the fact that missense mutations may disrupt the functional amino acid networks described above. Based on this, we propose a general model where single amino acid substitutions lead to global conformational changes with misfolding that is communicated throughout the whole protein (Fig. 5).

There is a growing appreciation of the role of biophysical properties of proteins in health and in disease (DePristo et al. 2005; Ignatova 2005; Luheshi and Dobson 2009). The thermodynamic stability of a protein is a determinant of the ratio of folded to unfolded molecules. Protein evolution led to a very narrow window of optimized free energy (ΔG) allowing for both reasonable stability and sufficient flexibility (Fig. 6). Thus, the maximum protein fitness mirrors the trade-off between stability, aggregation, degradation, and function of a given protein (DePristo et al. 2005). The restricted thermodynamic range explains the fact that proteins are in a fragile balance that can easily be disturbed by changes in external and internal factors such as missense mutations. Moreover, this clarifies that minor local changes in the structure caused by single amino acid replacements lead to undue global rearrangements and general dysfunction of the protein.

Examining a protein's folding rate, misfolding rate, and stability, there is a so-called proteostasis boundary, where proteins are in an equilibrium so that they can function normally (Fig. 7). Mutations can alter the folding energetics of the protein, which leads to changes in folding and misfolding rates and in stability. The protein now falls outside the proteostasis boundary. This can lead to either loss of function or to aggregation. Pharmacological chaperones

bind to the protein and improve its folding energetics. This rescues the protein's function by increasing the folding rate, decreasing the misfolding rate, increasing the stability, or any combination thereof (Powers et al. 2009).

In summary, we note the following:

- Single amino acid side chains can be involved in several protein functions.
- Single amino acid side chains can be involved in structural and functional networks through structural interactions.
- Single amino acid replacements lead to global structural rearrangements and to functional impairment.
- The window of thermodynamic stability for optimal function is narrow.
- Pharmacological chaperones can rescue protein function, mainly by increasing protein stability.

These findings support the hypothesis that virtually all missense mutations may lead to protein misfolding with loss of function.

The vision of a systematic approach to detect genetic (and nongenetic) protein misfolding disorders with loss of function and to develop pharmacological chaperone therapies for patients suffering from these disorders

If it holds true that protein misfolding with loss of function is a general molecular consequence of missense mutations, and if we take into account that the overall frequency of monogenetic diseases is 1 in 100 with a total of 100,329 disease-associated single amino acid substitutions described and 60% being missense or small deletions/insertions (Cooper et al. 2010; Zhong et al. 2009), a systematic approach to detect genetic protein misfolding disorders and to develop pharmacological chaperone therapies for these diseases may be promising. This concept could lead to a class of next generation orphan drugs for patients with inborn errors of metabolism, for other monogenetic diseases, for polygenetic diseases, and potentially also for nongenetic multifactorial diseases.

Epilogue

In conclusion, 56 years ago Horst Bickel exploited a new technical opportunity to remove single amino acids from a protein hydrolysate to develop the first dietary treatment for a previously untreatable genetic disease. This significantly changed daily life of patients suffering from phenylketonuria and other inborn errors of metabolism. Recently, a second paradigm change was achieved with the approval of the first pharmacological treatment for PAH deficiency. Again, technological advances promoted an in-depth understanding of the molecular pathophysiology linked to *PAH* mutations and of the mechanisms that rescue the molecular phenotype. This led to the hypothesis of a general molecular concept of protein misfolding linked to virtually all missense mutations that will define a new and rapidly expanding class of disorders and open the door to the development of next generation orphan drugs for patients with inborn errors of metabolism and other genetic and nongenetic diseases.

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