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MINIREVIEW ARTICLE

Protein homeostasis disorders of key enzymes of amino acids metabolism: mutation-induced protein kinetic destabilization and new therapeutic strategies

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Abstract Many inborn errors of amino acids metabolism are caused by single point mutations affecting the ability of proteins to fold properly (i.e., *protein homeostasis*), thus leading to enzyme loss-of-function. Mutations may affect protein homeostasis by altering intrinsic physical properties of the polypeptide (folding thermodynamics, and rates of folding/unfolding/misfolding) as well as the interaction of partially folded states with elements of the protein homeostasis network (such as molecular chaperones and proteolytic machineries). Understanding these mutational effects on protein homeostasis is required to develop new therapeutic strategies aimed to target specific features of the mutant polypeptide. Here, I review recent work in three different diseases of protein homeostasis associated to inborn errors of amino acids metabolism: phenylketonuria, inherited homocystinuria and primary hyperoxaluria type I. These three different genetic disorders involve proteins operating in different cell organelles and displaying different structural complexities. Mutations often decrease protein kinetic stability of the native state (i.e., its half-life for irreversible denaturation), which can be studied using simple kinetic models amenable to biophysical and biochemical characterization. Natural ligands and pharmacological chaperones are shown to stabilize mutant enzymes, thus supporting their therapeutic application to overcome protein kinetic destabilization. The role of molecular chaperones in protein folding and misfolding is also discussed as well as their potential pharmacological modulation as promising new therapeutic approaches. Since current available treatments for these diseases are either burdening or only successful in a fraction of patients, alternative treatments must be considered covering studies from protein structure and biophysics to studies in animal models and patients.

Keywords Amino acid metabolism · Protein homeostasis · Conformational disease · Protein stability · Ligand binding · Pharmacological therapy

Abbreviations

AGT	Alanine:glyoxylate aminotransferase
CBS	Cystathionine β-synthase
PAH	Phenylalanine hydroxylase
PH1	Primary hyperoxaluria type I
PKU	Phenylketonuria
HC	Inherited homocystinuria
PLP	Pyridoxal 5′-phosphate
BH_4	Tetrahydrobiopterin
DSC	Differential scanning calorimetry
SAM	*S*-adenosyl-L-methionine

A brief overview of protein folding in vivo: the protein homeostasis network

Evolution has provided living organisms with complex and highly regulated systems to preserve efficient protein folding in vivo and to adapt to diverse challenges such as mutations and environmental stresses thus promoting organism survival (Balch et al. 2008; Powers et al. 2009; Powers and Balch 2013). Over 800 different proteins are

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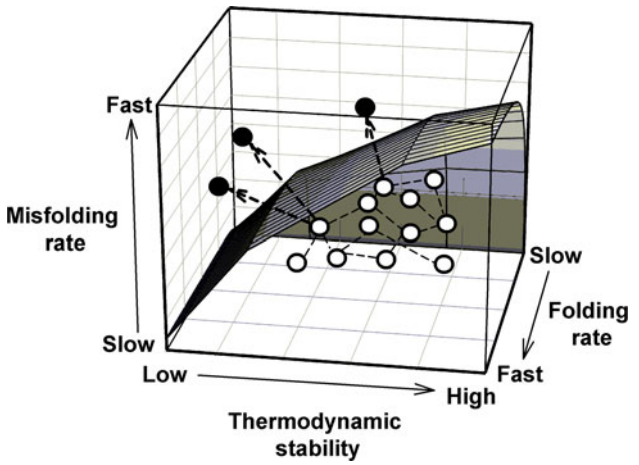

Fig. 1 Schematic representation of a protein homeostasis boundary. The three-dimensional coordinates for a protein are provided by different set of values of energetic properties (in this case, thermodynamic stability, folding and misfolding rates). The *empty circles* represent different sets of values given by different intracellular conditions (e.g. protein–ligand interactions or different levels of molecular chaperones) that fall below the proteostasis boundary (which defines the sets of values compatible with protein folding and function in health). However, different challenges (such as mutations or oxidative stress) may change this set of values beyond the proteostasis boundary, leading to disease. This figure is inspired by Powers et al. (2009)

implicated in this complex *protein homeostasis network* responsible for protein synthesis, folding, trafficking and degradation, which is organized in different compartment-specific pathways (Powers et al. 2009; Calamini et al. 2011; Hartl et al. 2011; Powers and Balch 2013). Protein homeostasis balance is also maintained by compartment-specific stress response systems including the heat shock response (HSR) for cytosolic proteins, the unfolded protein response (UPR) in the endoplasmic reticulum and mitochondria, or the antioxidant response (ARE) (Calamini et al. 2011; Powers and Balch 2013). Loss of protein homeostasis control is associated to aging and many folding disorders, including neurodegenerative and metabolic diseases (Balch et al. 2008; Martinez et al. 2008; Powers et al. 2009; Ong and Kelly 2011).

Protein folding efficiency in vivo depends on a balance between protein physical properties such as thermodynamic stability and folding/unfolding/misfolding rates, as well as on the interaction of different protein states along folding and misfolding with the protein homeostasis network (Balch et al. 2008; Powers et al. 2009). The relationships between all these aspects of intracellular protein folding have been conceptually integrated in the *protein homeostasis boundaries* (Wiseman et al. 2007; Powers et al. 2009). The protein homeostasis boundary is a surface defined by a set of coordinates for energetic parameters of a given protein (in terms of folding thermodynamics, unfolding/folding/misfolding rates) that are consistent with its proper folding intracellularly (see Fig. 1 for an example). One can anticipate that many proteins under healthy physiological conditions must display energetic parameters compatible with efficient folding and function (the open symbols in Fig. 1). The intracellular milieu also modulates these energetic parameters: molecular chaperones may decrease misfolding and/or accelerate folding rates and native state ligands may decrease unfolding rates and/or enhance thermodynamic stability (this modulation is displayed by the different open symbols in Fig. 1). Environmental (e.g. temperature, oxidative stress, aging) and genetic (e.g. mutations and polymorphisms) factors may challenge protein foldability by placing protein energetics beyond the proteostasis boundary, therefore leading to disease (shown as black symbols in Fig. 1; Powers et al. 2009). Actually, individual differences in protein homeostasis capacity exist among (even isogenic) individuals thus yielding different phenotypes for the same genotype (Casanueva et al. 2012). Thus, epistatic, epigenetic and environmental factors also play a role in protein homeostasis, which may explain the difficulty of establishing genotype–phenotype correlations in many human misfolding diseases (Casanueva et al. 2012; Lehner 2013).

Thermodynamic vs. kinetic stability of proteins

As indicated above, physicochemical properties such as protein thermodynamic and kinetic stabilities play an important role in protein homeostasis. The concept of thermodynamic stability is introduced here using the simplest case, the two-state equilibrium model. This model is an extreme simplification of protein folding but it is suitable to introduce some basic relationships that exist between thermodynamic and kinetic stabilities (Plaza del Pino et al. 2000; Sanchez-Ruiz 2010). The two-state equilibrium model is depicted by:

$$N \overset{K_U}{\leftrightarrow} U \quad \text{(model 1)}$$

where, N stands for the native state and U for the unfolded state (or an ensemble of unfolded states), and implies that additional intermediate unfolded states are thus very unstable, and not significantly populated at equilibrium. In this simple scenario, thermodynamic stability is determined by the equilibrium unfolding constant $K_U = [U]/[N]$, which is related to the standard unfolding free energy change by $\Delta G = G_U - G_N = -RT \cdot \ln K_U$. Thus, thermodynamic stability determines the fraction of unfolded protein at equilibrium. The unfolding free energy at ~physiological temperatures is often small, usually in the range of a -2 to -15 kcal mol^{-1}

(Dill et al. 2008; Nordlund and Oliveberg 2008; Gomes 2012), hence, leading to fractions of unfolded state as high as 3.3 %. Hence, protein native states are often marginally stable from a thermodynamic perspective. We must also note that the unfolding equilibrium is dynamic and displays kinetic features, since $K = k_U/k_F$, where k_U and k_F are the unfolding ($N \rightarrow U$) and folding ($U \rightarrow N$) rate constants.

Nevertheless, (partially) unfolded states are often prone to aggregation or degradation in vitro and in vivo, and thus, protein denaturation is not reversible and equilibrium thermodynamics may not be applicable (Plaza del Pino et al. 2000; Powers and Balch 2013). In vitro, these irreversible phenomena will deplete the unfolded state in a time-dependent manner, thus shifting the $N \leftrightarrow U$ equilibrium until the native state has been completely consumed. Hence, unfolding thermodynamics does not necessarily dictates the relevant kinetic stability of the protein (i.e., the time-scale of irreversible denaturation, protein shelf- or half-life) under certain given conditions (Plaza del Pino et al. 2000). In fact, it has been shown that thermodynamic stability does not guarantee the safety of the native state and, conversely, a thermodynamically unstable protein may be kinetically robust (Plaza del Pino et al. 2000; Sanchez-Ruiz 2010). Indeed, a large kinetic stability may be achieved by a very slow unfolding (i.e., large unfolding activation free energy), a mechanism which is dramatically exemplified by the case of the α-lytic protease (Baker et al. 1992; Baker and Agard 1994).

Some irreversible processes (such as aggregation) are expected to occur fast in vivo, at least partly due to the high concentration of background macromolecules (macromolecular crowding, Hartl and Hayer-Hartl 2009; Sanchez-Ruiz 2010). Protein irreversible denaturation is often interpreted by a Lumry–Eyring model (model 2; Lumry and Eyring 1954), which describes well the irreversible denaturation of many proteins (Park and Marqusee 2004; Sanchez-Ruiz 2010; Pey et al. 2013a):

$$N \overset{K_U}{\leftrightarrow} U \overset{k_{IRR}}{\longrightarrow} F \quad \text{(model 2)}$$

where the U state undergoes an irreversible process to reach a final state F, and this process is characterized by a rate constant k_{IRR}. In many cases, the U state is not significantly populated (i.e., its concentration is much lower than for N and F) along irreversible denaturation (Sánchez-Ruiz et al. 1988; Sanchez-Ruiz 2010; Pey et al. 2011, 2013a, b; Mesa-Torres et al. 2013) and hence, model 2 becomes phenomenologically described by a simple two-state kinetic model:

$$N \overset{k}{\longrightarrow} F \quad \text{(model 3)}$$

where k is the first-order rate constant for overall denaturation, and the protein half-life is simply estimated as $t_{1/2} = \ln 2/k$. In this scenario, protein kinetic stability is determined by the height of the free energy barrier that the native state must cross to reach the transition state of the rate-limiting step of denaturation (Sanchez-Ruiz 2010; Salido et al. 2012). Note that this rate for irreversible denaturation (due to aggregation or proteolytic cleavage) is conceptually similar to a biologically relevant misfolding rate contributing to the proteostasis boundary (Fig. 1) since it may resemble a relevant stability of the native state in vivo.

Early work from Sanchez-Ruiz and coworkers provided several tests (Sánchez-Ruiz et al. 1988) to determine whether this simple kinetic model (model 3) describes well thermal denaturation scans of proteins. Figure 2a–d shows these tests applied on wild-type human alanine:glyoxylate aminotransferase based on differential scanning calorimetry (DSC) analyses (Pey et al. 2011; Sanchez-Ruiz 2010; Salido et al. 2012). Analysis of DSC experiments provides the value of k (or the half-life) directly from the thermal scan at several temperatures (Sánchez-Ruiz et al. 1988). These DSC analyses are equivalent to perform enzyme inactivation experiments at multiple temperatures, but with the advantage of providing robust values of k at different temperatures from a few scans and several tests to support the adequacy of this model.

There are two limiting situations consistent with the two-state kinetic model (model 3): (A) the irreversible step is fast compared to the folding step ($k_{IRR} \gg k_F$), and thus, the overall denaturation rate (k) is determined by unfolding kinetics (k_U); (B) the irreversible step is rate-limiting ($k_{IRR} \ll k_F$) and thus, $k = K \cdot k_{IRR}$ (see Plaza del Pino et al. 2000; Pey et al. 2013a for a detailed discussion). Therefore, what makes the difference between situations A and B is whether the unfolded state *prefers* to undergo the irreversible step (A) or to refold (B). Moreover, situation A clearly shows why the kinetic stability of a protein may not be linked to its thermodynamic stability (which is further discussed for the case of human alanine:glyoxylate aminotransferase (AGT) below; Fig. 2). More complex models for irreversible denaturation of proteins may also include the effect of oligomerization (i.e., non first-order kinetics) and ligand binding on the rates of irreversible denaturation using comparatively simple theoretical and experimental approaches (Figs. 2, 3, for human AGT and CBS enzymes; see Sanchez-Ruiz 1992; Pey et al. 2011, 2013b; Sánchez-Romero et al. 2013 for further description).

The role of protein kinetic stability in genetic disorders of amino acids metabolism and its relationship with protein homeostasis defects

In this section, three genetic disorders of protein homeostasis affecting different key enzymes of amino acids

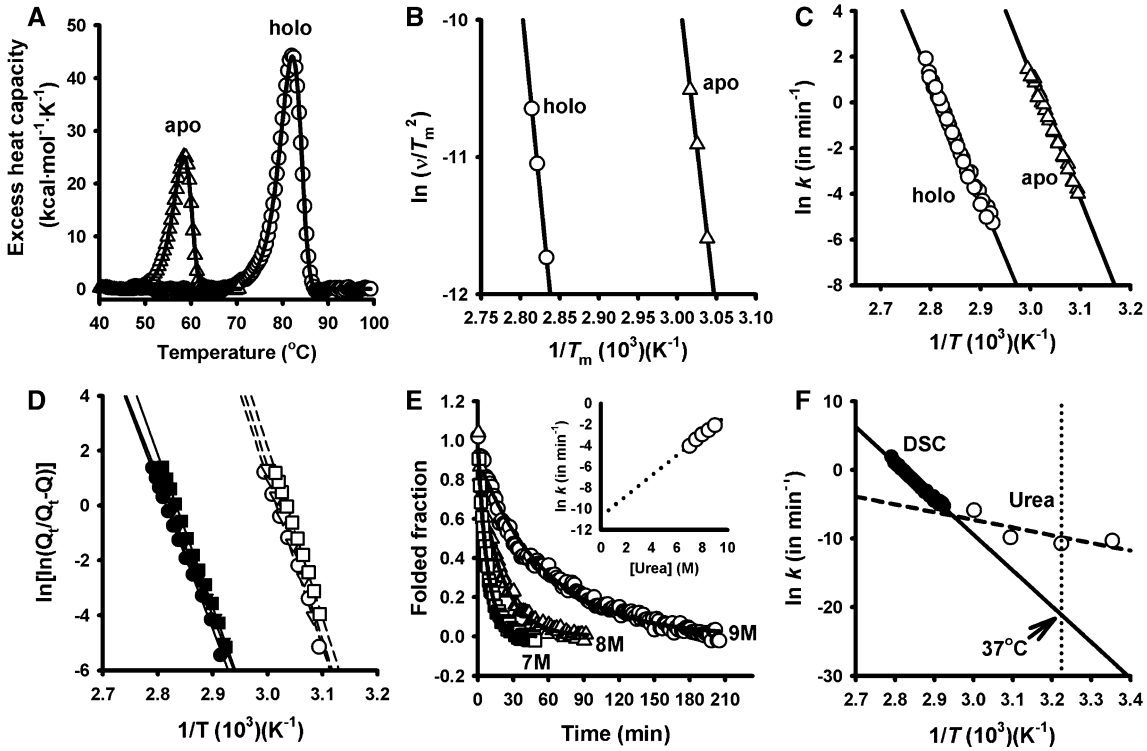

Fig. 2 Thermal and kinetic stability of human AGT WT. **a** DSC scans of holo- and apo-AGT; **b–d** consistency tests proposed by Sánchez-Ruiz et al. (1988), supporting the applicability of a two-state kinetic model to describe AGT thermal denaturation. **e** Unfolding kinetics of holo-AGT at 37 °C at different urea concentrations measured by Far-UV circular dichroism spectroscopy. *Inset* linear extrapolation of unfolding rates to no denaturant. **f** Comparison of thermal and urea denaturation of holo-AGT supporting that at physiological temperature, AGT kinetic stability is not rate-limited by global unfolding kinetics. The *vertical dotted line* indicates physiological temperature

metabolism are presented: primary hyperoxaluria type I (PH1), inherited homocystinuria (HC) and phenylketonuria (PKU). We will focus on the mutational effects on protein kinetic stability. For these three disorders, protein kinetic destabilization is likely associated with altered patterns of interaction with elements of the protein homeostasis network, contributing to enzyme loss-of-function (a brief compilation of these alterations in protein homeostasis can be found in the SI text).

Human alanine:glyoxylate aminotransferase (AGT) and primary hyperoxaluria type I (PH1)

Human AGT is a pyridoxal-5′phosphate (PLP) dependent enzyme that catalyzes the transamination of L-Ala to glyoxylate to form pyruvate and glycine in liver peroxisomes (Danpure 2001, 2006; Cellini et al. 2011; Salido et al. 2012) (Figure S1A-B). Human AGT forms active 86 kDa dimers and each AGT monomer contains two domains, a N-terminal catalytic domain containing the substrate and PLP binding sites, and a C-terminal domain containing the peroxisomal targeting sequence (PTS; Figure S1C). AGT is responsible for glyoxylate detoxification in humans (Figure S1B), and its failure due to mutations causes PH1. PH1 leads to a glyoxylate build-up, and its subsequent oxidation to oxalate (which is a metabolic end-product in humans) eventually causes renal failure owing to calcium oxalate deposition (Figure S1B and Danpure 2001, 2006; Salido et al. 2012). A combined double liver and kidney transplantion increase patients life-span and quality of life, even though some patients also respond positively to supplementation with the PLP precursor, pyridoxine, and often only require kidney transplantation (Salido et al. 2012). From a genetic viewpoint, about half of the mutations occur in a polymorphic background containing two amino acid substitutions (the minor haplotype, or AGT-LM) respect to the more frequent major haplotype (AGT-WT), which is not disease causing by itself but sensitizes AGT towards additional mutations (Danpure 2001, 2006; Cellini et al. 2011; Salido et al. 2012; Fargue et al. 2013a). Over 150 mutations have been described in PH1 patients, and a large fraction of them are missense mutations (recently summarized in Williams et al. 2009). From a cell biology viewpoint, PH1 is also a very interesting model to understand the pathways involved in protein homeostasis and intracellular trafficking, because different mutations either

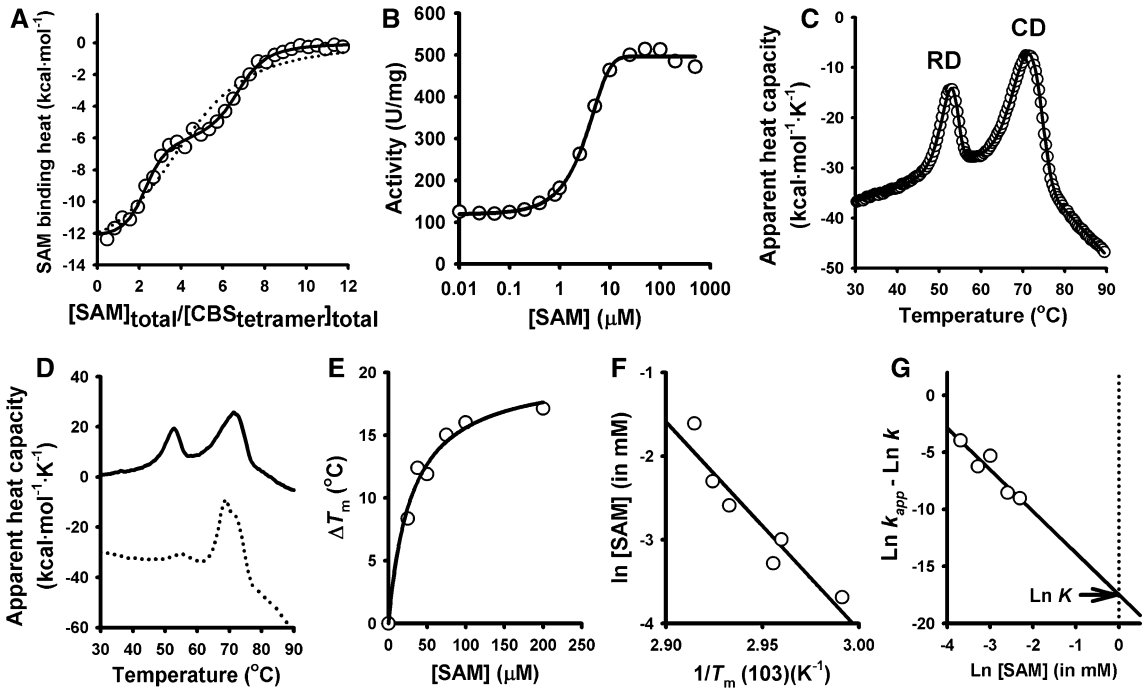

Fig. 3 Thermal stability and SAM binding to WT human CBS. **a, b** titration with SAM by isothermal titration calorimetry (ITC; **a**) and activity (**b**) of human CBS at 25 °C; the *solid line* in **a** is a fit to two independent type of sites, while the *dotted line* is a fit to a single type of sites; **c** thermal denaturation by differential scanning calorimetry (DSC). *RD* and *CD* refer to the denaturation of regulatory and catalytic domains. The *solid line* is a fit to two independent two-state kinetic transitions with first-order kinetics. **d** DSC experiments in the absence (*solid line*) or the presence of 100 μM SAM (*dotted line*) showing specific stabilization of RDs. **e** SAM concentration dependence of thermal up-shift of RDs. **f** analysis of SAM mediated stabilization by a phenomenological two-state kinetic model (the slope of this plot is equal to $-E_a/(v \cdot R)$, where v is the number of SAM molecules released prior to the denaturation rate-limiting step and E_a is the activation energy); **g** analysis of SAM mediated stabilization by a kinetic mechanism (the slope of this plot is equal to $-v$ and the y-intercept is ln K, where the square root of K is the affinity constant of those sites involved in SAM mediated stabilization. For further details, see Pey et al. (2013b)

cause normal import to peroxisomes but as inactive aggregates or mitochondrial mistargeting (where the enzyme is metabolically inefficient) (Salido et al. 2012; Fargue et al. 2013b; Mesa-Torres et al. 2013). PH1 mutations may affect AGT folding and stability in different ways, including low protein kinetic stability of the apo-forms (with no PLP bound; Cellini et al. 2010a, 2011; Salido et al. 2012; Mesa-Torres et al. 2013) and strong interactions with molecular chaperones of the Hsp60, 70 and 90 families (Santana et al. 2003; Albert et al. 2010; Pey et al. 2011; Mesa-Torres et al. 2013). Thus, the partition of AGT variants between folding into dimers and peroxisomal targeting, engagement with the mitochondrial import machinery and mistargeting, and aggregation seems to rely on a delicate balance between protein kinetic stability and interaction of partially folded states with protein homeostasis pathways, which might be therapeutically targeted to restore AGT function in PH1 patients (see SI text and Pey et al. 2011; Salido et al. 2012; Mesa-Torres et al. 2013).

Even though crystal structures and structure-based computational procedures have allowed to rationalize the effects of some mutations on AGT function (Cellini et al. 2011; Oppici et al. 2012, 2013; Mesa-Torres et al. 2013), in most of the cases they have not been able to predict the impact of mutations on intracellular protein foldability. We have recently carried out exhaustive experimental studies on the relationship between mutational effects on protein kinetic stability and intracellular foldability of human AGT (Mesa-Torres et al. 2013). PLP binding to WT AGT and to most of the disease-causing mutants provides a tremendous stabilizing effect, increasing by ~25 °C their thermal stability (See Fig. 2a Salido et al. 2012). Since AGT denaturation is kinetically controlled (follows model 3), apo-proteins are kinetically destabilized by ~5–6 orders of magnitude at physiological temperature compared to holo-proteins (Fig. 2b Pey et al. 2011; Salido et al. 2012; Mesa-Torres et al. 2013). Despite the good agreement between mutational effects on the kinetic stability of holo- and apo-forms for each variant (Mesa-Torres et al. 2013), several PH1 causing mutants on the minor allele display even lower kinetic stability than the AGT-LM enzyme as apo-proteins, and this overstabilization by PLP cannot be explained by an increased binding affinity for PLP (Pey et al. 2011; Salido et al. 2012; Mesa-Torres et al. 2013). Actually, we found that

mutants with a decreased kinetic stability of the apo-form also largely reduced intracellular protein foldability (decreasing total expression levels and solubility in CHO cells), suggesting a link between these two phenomena (Mesa-Torres et al. 2013). Interestingly, two of these mutants (G170R and F152I on the minor allele) are known to be responsive to pharmacological doses of pyridoxine (van Woerden et al. 2004; Monico et al. 2005), which might be explained by an improvement of protein kinetic stability and intracellular foldability upon raising the intracellular concentration of PLP (Fargue et al. 2013a; Mesa-Torres et al. 2013).

Despite the inherent complexity expected for folding/unfolding of a protein of this size, AGT thermal and kinetic stability is described well by a simple two-state kinetic model (model 3; Pey et al. 2011; Salido et al. 2012; Mesa-Torres et al. 2013), while chemical denaturation by urea, guanidium and pH support the population of different unfolding intermediates with a large tendency to aggregate (Cellini et al. 2010b; Pey et al. 2011). Structure-energetic calculations indicate that thermal unfolding of AGT enzymes involves a large loss of tertiary structure, possibly owing to denaturation of both domains in the AGT monomer (Pey et al. 2011; Salido et al. 2012; Mesa-Torres et al. 2013). However, since denaturation follows first-order kinetics (i.e., it is independent on protein concentration), dimer dissociation must occur after the rate-limiting step of irreversible denaturation (Mesa-Torres et al. 2013). Urea unfolding rates for holo-AGT WT extrapolated to zero denaturant concentration (no denaturant) (Fig. 2e, f) suggest that global unfolding is faster than irreversible denaturation (from DSC) at physiological temperature. Hence, irreversible denaturation of WT AGT depends on both thermodynamic and kinetic aspects of unfolding (situation B in models 2–3), since global denaturation kinetics are not rate-limiting (see Sect. 2, and Plaza del Pino et al. 2000; Sanchez-Ruiz 2010; Pey et al. 2013a).

Human cystathionine β-synthase (CBS) and inherited homocystinuria (HC)

Human CBS is a ~250 kDa homotetramer that catalyzes the condensation of L-Serine and homocysteine to form cystathionine (Figure S2A). This reaction is one of the two key points of regulating methionine consumption, and depends on the activation of human CBS upon binding of *S*-adenosyl-methionine (SAM), thus committing methionine to the transulfuration pathway (Figure S2A). Each CBS monomer contains three domains: a N-terminal heme-containing domain, a central catalytic domain containing one molecule of reactive PLP and a C-terminal regulatory domain containing potentially two binding sites for the allosteric activator SAM (Figure S2B; Miles and Kraus 2004; Pey et al. 2013b). Over 160 mutations in human CBS cause HC (http://medschool.ucdenver.edu/krauslab), a disorder of sulfur aminoacid metabolism associated to mental retardation, cardiovascular and connective issues, among other symptoms (Mudd et al. 2001). Treatment of HC includes methionine restriction and supplementation with pyridoxine and betaine (Wilcken and Wilcken 1997; Yap et al. 2000, 2001). Missense mutations causing HC often perturb protein folding and stability (Majtan et al. 2010; Hnízda et al. 2012; Pey et al. 2013b). Actually, kinetic stability analyses of disease-causing mutants support that some of them reduce the stability of the regulatory domain, which might accelerate mutant CBS protein turnover in vivo (Prudova et al. 2006; Pey et al. 2013b). The effect of some HC mutations have been rationalized based on the crystal structure of a C-terminal truncated form of human CBS (Meier et al. 2003; see Figure S2C, supporting that mutations affecting buried residues are more often associated to severe misfolding (Kozich et al. 2010).

The intracellular stability and protein levels of human CBS are known to be modulated by SAM binding (Prudova et al. 2006); even though the structural and energetic basis of SAM mediated activation and stabilization have remained unclear. We have recently applied a strategy combining calorimetric, spectroscopic and functional assays to dissect the role of SAM as an allosteric activator and native state kinetic stabilizer of human CBS (Pey et al. 2013b). Direct calorimetric titrations of human CBS WT and disease-causing mutants show a complex binding pattern (Fig. 3a) supporting the existence of two types of SAM binding sites rather than a single type of sites: a high affinity type of sites (K_d ~ 10 nM, and two SAM molecules bound/tetramer) and a low affinity type of sites (K_d ~ 0.5 μM, and four SAM molecules bound/tetramer). SAM binding to low affinity sites is consistent with ligand-mediated activation of the WT enzyme (Fig. 3b) but the role of high affinity sites was not evident at first sight. Interestingly, some HC mutants known to abolish SAM mediated regulation of CBS showed similar binding pattern than WT CBS, suggesting that the allosteric signal responsible for CBS activation must be subtle in structural and energetic terms (Pey et al. 2013b). DSC thermal denaturation analyses showed that regulatory and catalytic domains denature independently and follow a simple phenomenological two-state kinetic model with first-order kinetics (model 3, Fig. 3c) and their kinetic stabilities at physiological temperature largely differed, with half-lives of ~1 day and ~3 weeks, respectively (Pey et al. 2013b). Mutations in the catalytic core caused kinetic destabilization of regulatory domains, suggesting some degree of communication between domains (Pey et al. 2013b). SAM binding specifically stabilizes the regulatory domains in a

concentration dependent manner (Fig. 3d, e). Interestingly, the half-life for denaturation of the regulatory domain determined by DSC is close to the turnover of CBS in cell cultures with low SAM intracellular levels ($t_{1/2}$ of 18 h), while a rise in SAM concentration slows down intracellular turnover by ~2.5-fold (Prudova et al. 2006). These results support a link between the kinetic stability of regulatory domains and intracellular half-life of human CBS (Pey et al. 2013b). Kinetic stabilization induced by SAM in WT and disease-causing CBS enzymes has been further analyzed by a simple two-state kinetic model (Fig. 3f), supporting that ~2 mol of SAM is responsible for kinetic stabilization of regulatory domains in the CBS tetramer. Further modeling of SAM-induced stabilization using a kinetic mechanism confirmed that ~2 mol of SAM is responsible for ligand-mediated stabilization of regulatory domains (Fig. 3g; Pey et al. 2013a, b). The K_d values provided by this model for these sites ($K_d \leq 20$ nM; Fig. 3g; Pey et al. 2013b) are consistent with those sites of high affinity obtained from direct titrations with SAM (Fig. 3a). The independent modulation of the activity and stability of human CBS also suggests that we might develop kinetic stabilizers targeting the high affinity sites without compromising allosteric activation of the enzyme by SAM (Pey et al. 2013b).

Human phenylalanine hydroxylase (PAH) and phenylketonuria (PKU)

Human PAH is the iron-containing enzyme catalyzing L-Phe to L-Tyr hydroxylation in the presence of tetrahydrobiopterin (BH_4) as cofactor in the cytosol of hepatocytes (Flydal and Martinez 2013) (Figure S3A). Human PAH forms active tetramers and dimers, and each monomer (~52 kDa) contains three structural domains: a regulatory N-terminal domain, responsible of L-Phe/BH_4 mediated allosteric regulation of PAH activity and also containing a site for phosphorylation (Ser16); a central catalytic domain, containing the catalytic iron and the binding sites for L-Phe and BH_4, and a C-terminal domain involved in oligomerization (Flydal and Martinez 2013) (Figure S3B-C). About 600 mutations in the PAH protein are associated to phenylketonuria (PKU) (http://www.pahdb.mcgill.ca/). PAH deficiency causes a build-up of plasma phenylalanine, eventually leading to mental retardation if untreated using a low-Phe diet (Underhaug et al. 2012). Alternative treatments based on BH_4 supplementation have been successful, at least in the milder phenotypes (Pey and Martinez 2007; Muntau and Gersting 2010; Underhaug et al. 2012). PKU is a paradigm of loss-of-function folding diseases (Pey et al. 2007; Gersting et al. 2008; Martinez et al. 2008; Gomes 2012; Underhaug et al. 2012), and most of the disease-causing mutations affect PAH thermal stability, its ability to fold into functional tetramers and enhance protein degradation and oxidative inactivation (Gamez et al. 2000; Pey et al. 2003, 2004a, 2007; Gersting et al. 2008; Martinez et al. 2008).

Human PAH activity is tightly regulated by multiple mechanisms, including substrate and cofactor binding, and phosphorylation at the Ser16 (excellent reviews for PAH structure, regulation, evolution and catalytic mechanism have been recently published by Fitzpatrick (2012) and Flydal and Martinez (2013). This tight control is required to maintain an adequate balance between L-Phe degradation and maintaining L-Phe and L-Tyr availability for anabolic processes (Flydal and Martinez 2013) (Figure S3A). PAH seems to exists in hepatocytes forming a 1:1 complex with BH_4 (per monomer) at low-Phe levels, ready to be activated by a raise in L-Phe levels, suggesting the existence of two pools of BH_4 with different modes of action (one inhibiting/stabilizing PAH, one available for catalytic turnover; Mitnaul and Shiman 1995). High L-Phe levels activate the enzyme (~3- to 4-fold for human PAH; Miranda et al. 2002; Thórólfsson et al. 2003; Erlandsen et al. 2004) also displaying positive cooperativity by shifting the equilibrium between low- and high-activity enzyme states (Flydal and Martinez 2013). The conformational changes occurring in human PAH upon L-Phe and BH_4 binding have been studied in some detail by molecular dynamic simulations and biophysical methods (Thórólfsson et al. 2003; Pey et al. 2004b). These studies support intertwined effects on the tertiary and quaternary structures of PAH upon binding of these ligands, likely involving large changes in conformation of the regulatory domain and in the accessibility of the active site (Flydal and Martinez 2013). Despite L-Phe and BH_4 seem to bind exclusively to the catalytic domains, both ligands are able to stabilize towards thermal denaturation both the regulatory and catalytic domains possibly by propagation of the stabilizing effect between domains (Thórólfsson et al. 2002; Erlandsen et al. 2004). BH_4-mediated stabilization seems to be especially relevant to overcome the intrinsically low kinetic stability of the regulatory domains, at least in vitro (Martinez et al. 2008; Dobrowolski et al. 2009). Nevertheless, the role of L-Phe and BH_4 on the in vivo activity, stability and turnover of hPAH seems to be quite complex (Mitnaul and Shiman 1995; Gersting et al. 2010; Sarkissian et al. 2012).

Thermal denaturation of human PAH is irreversible, and catalytic and regulatory domains unfold independently (Thórólfsson et al. 2002; Erlandsen et al. 2004; Gersting et al. 2008). PKU mutants often decrease PAH thermal stability, owing to the destabilization of regulatory domains in many cases (Gamez et al. 2000; Pey et al. 2003; Gersting et al. 2008; Dobrowolski et al. 2009). Large-scale structure-based computational analyses using the Fold-X force

field have proposed an almost universal native state destabilization by PKU mutations, with a potential direct translation on the relevant thermodynamic and kinetic stabilities of PAH which may underlie folding and stability defects in PKU (Pey et al. 2007). Pharmacological treatment of PKU patients with BH_4 lead to correction of the metabolic phenotype thorough the kinetic stabilization of the PAH tetramer among other mechanisms (Erlandsen et al. 2004; Pey et al. 2004a; Pey and Martinez 2007; Martinez et al. 2008). Alternatively to BH_4, native state kinetic stabilizers have been also discovered as pharmacological chaperones for PKU from experimental and virtual screening of chemical libraries (recently reviewed by Underhaug et al. 2012).

Native state kinetic stabilizers as plausible therapies: natural and pharmacological chaperones

Specific ligand binding to the native state of a protein is expected to increase its kinetic stability if the ligand does not bind to the transition state of the denaturation rate-limiting step (based on model 3; Sanchez-Ruiz 1992; Pey et al. 2013b). In other words, native state kinetic stabilization occurs owing to the preferential thermodynamic stabilization of the native state, which decreases the population of partially folded states prone to misfold (now, based on model 2). Therefore, ligand binding may overcome protein homeostasis defects by simply changing protein energetics (at kinetic or thermodynamic levels). This change in protein energetics may result in crossing the protein homeostasis boundary back to values compatible with protein foldability, thus reversing the misfolding loss-of-function phenotype (see Fig. 1; Powers et al. 2009). Indeed, many natural ligands might act in vivo as kinetic stabilizers (Pey and Martinez 2007; Rodrigues et al. 2012; Pey et al. 2013b). Alternatively, non-natural kinetic stabilizers may be also found by high-throughput screens of chemical libraries (e.g. by thermal up-shift assays; Pey et al. 2008; Underhaug et al. 2012). In this section, kinetic stabilizers for HC, PKU and PH1 are presented and discussed as potentially therapeutic strategies. We must note, however, that beyond their effect on kinetic stabilities they might also work as folding aids intracellularly by other mechanisms, as recently discussed in detail (Rodrigues et al. 2012). Unfortunately, the effects of ligands as folding aids in HC, PKU and PH1 are difficult to characterize in vitro due to the irreversible denaturation of the proteins causing these diseases.

PH1

Patients carrying certain PH1 mutations respond to pharmacological doses of pyridoxine (vitamin B6), the precursor of PLP (van Woerden et al. 2004; Monico et al. 2005). However, the levels and reactivity of PLP are tightly controlled in vivo by several enzymes regulating PLP synthesis from pyridoxine and its recycling, to ensure proper PLP transfer to apo-proteins and avoid undesirable side reactions with amine groups (recently reviewed by di Salvo et al. 2011). A plausible mechanism to explain pyridoxine responsive PH1 is the kinetic stabilization exerted by cofactor (especially in those apo-forms kinetically destabilized to a larger extent, such as F152I and G170R on the minor allele; Mesa-Torres et al. 2013) as well as changes in protein unfolding pathways (as suggested by chemical denaturation of AGT enzymes; Cellini et al. 2010a, b). Since PLP bioavailability depend in the pyridoxine dietary intake but also on the regulation of the enzymes responsible for synthesis, recycling and transfer of PLP to apo-proteins, alternative treatment might be envisioned based on the regulation of PLP converting/recycling enzymes as well as dietary supplementation with pyridoxine (Mesa-Torres et al. 2013).

HC

A significant fraction of HC patients also respond to pharmacological doses of pyridoxine (Mudd et al. 2001; Clayton 2006). Supplementation with pyridoxine leads to a 1.3- to 4.5-fold increase in CBS hepatic activity, supporting enhanced CBS activity and stability (Mudd et al. 2001; Clayton 2006). Moreover, the pyridoxine responsive R266K mutant causes a structural perturbation in its complex with PLP reducing its affinity for PLP (3- to 10-fold increase in K_M; (Chen et al. 2006; Smith et al. 2012)), which might also explain its responsiveness to pyridoxine due to an enhancement of enzyme activity and stability.

An activity-based platform for high-throughput screenings for chemical libraries aimed to identify inhibitors and kinetic stabilizers of human CBS has been recently presented, thus paving the way to develop specific pharmacological chaperones for HC (Thorson et al. 2013). Moreover, the recent discovery of high affinity sites for SAM responsible for kinetic stabilization of human CBS regulatory domain also support that pharmacological ligands may be developed to enhance human CBS kinetic stability without interfering with proper activation of the enzyme upon SAM binding to regulatory sites (Pey et al. 2013b).

PKU

Since 1999, when Kure and coworkers (Kure et al. 1999) described the positive response of several PKU patients to pharmacological doses of BH_4, there has been a world-

wide increasing interest in BH_4 supplementation as an alternative to Low-Phe diet in PKU patients, at least in those with milder phenotypes (Underhaug et al. 2012). The molecular basis of BH_4 responsiveness seems to be multifactorial, including BH_4 mediated protein stabilization (chaperone effect) (Erlandsen et al. 2004; Pey et al. 2004a, b; Martinez et al. 2008; Gersting et al. 2010; Sarkissian et al. 2012; Underhaug et al. 2012). Recent studies have also suggested that stimulation of PAH activity upon BH_4 treatment requires a complex and coordinated action of increased L-Phe and BH_4 levels on the activity landscape, intracellular stability and ubiquitin-dependent degradation of PKU mutants which seems to be mutant-specific (Gersting et al. 2010; Staudigl et al. 2011; Sarkissian et al. 2012).

BH_4 has been recently used as a query to identify alternative kinetic stabilizers of human PAH by structure-based virtual screening (Santos-Sierra et al. 2012). Several classes of compounds were found to bind to human PAH with affinities similar to that of BH_4, and their efficacy as kinetic stabilizers was confirmed by biochemical and biophysical procedures. Administration of these new pharmacological chaperones to suitable animal models of PKU was also shown to provide even better improvement of L-Phe hydroxylation capacity than BH_4 (Santos-Sierra et al. 2012).

Upon screening of a commercial chemical library, we identified four compounds that bound to human PAH (Pey et al. 2008) using a thermal up-shift procedure. These compounds acted also as kinetic stabilizers of WT and mutant PAH enzymes, with affinities similar to BH_4. Administration of these compounds to cultured cells expressing different PKU variants showed a ligand- and mutant-specific positive response. Moreover, two of these compounds were found to enhance protein levels and activity in WT mice, confirming other studies performed with BH_4 supporting that kinetic stabilizers may increase WT PAH protein levels in vivo (Thöny et al. 2004; Scavelli et al. 2005; Gersting et al. 2010; Sarkissian et al. 2012).

Concluding remarks

The intention of this minireview was to provide a brief description of protein homeostasis defects and the role of protein kinetic stability in loss-of-function diseases, particularly on three disorders associated to key enzymes of amino acid metabolism. This allowed us to introduce and rationalize current and potentially new treatments based on protein kinetic stabilization and modulation of protein homeostasis for these genetic diseases, in the hope that the ideas and works presented and discussed here will be insightful for researchers studying other genetic disorders of amino acids metabolism.

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