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Minireview

New protein structures provide an updated understanding of phenylketonuria

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ABSTRACT

Phenylketonuria (PKU) and less severe hyperphenylalaninemia (HPA) constitute the most common inborn error of amino acid metabolism, and is most often caused by defects in phenylalanine hydroxylase (PAH) function resulting in accumulation of Phe to neurotoxic levels. Despite the success of dietary intervention in preventing permanent neurological damage, individuals living with PKU clamor for additional non-dietary therapies. The bulk of disease-associated mutations are PAH missense variants, which occur throughout the entire 452 amino acid human PAH protein. While some disease-associated mutations affect protein structure (e.g. truncations) and others encode catalytically dead variants, most have been viewed as defective in protein folding/stability. Here we refine this view to address how PKU-associated missense variants can perturb the equilibrium among alternate native PAH structures (resting-state PAH and activated PAH), thus shifting the tipping point of this equilibrium to a neurotoxic Phe concentration. This refined view of PKU introduces opportunities for the design or discovery of therapeutic pharmacological chaperones that can help restore the tipping point to healthy Phe levels and how such a therapeutic might work with or without the inhibitory pharmacological chaperone BH_4. Dysregulation of an equilibrium of architecturally distinct *native* PAH structures departs from the concept of "misfolding", provides an updated understanding of PKU, and presents an enhanced foundation for understanding genotype/phenotype relationships.

Contents

1. Phenylketonuria – a brief overview

Phenylalanine (Phe), one of the twenty common amino acids that are the building blocks of all protein, cannot be synthesized by humans and must be obtained through diet and/or protein catabolism. Regardless of intake, humans generally maintain blood (plasma) Phe in a narrow range, and nearly always below 120 μM. Individuals who fail to do so have disorders ranging from hyperphenylalaninemia (HPA, 120–360 μM Phe) to the most severe forms of phenylketonuria (PKU, >1200 μM Phe) (OMIM 261600). Current treatment strategies focus on lowering Phe levels, though guidelines continue to evolve (e.g. [1–5]). Untreated PKU during brain development (infancy, childhood, adolescence) can result in profound and irreversible

Abbreviations: PAH, Phenylalanine hydroxylase; PKU, phenylketonuria.

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neurocognitive damage. Poor control of Phe levels, at any life stage, can result in failures in executive function or a need for psychological or psychiatric care [6]. Symptoms generally improve with reduction in blood Phe. Hence, lifelong control of blood Phe, through diet and/or pharmacological intervention, is the consensus recommendation for individuals living with HPA and PKU (hereafter together called PKU) [7].

PKU is the most common inborn error of amino acid metabolism, though frequency varies widely by country (~1:10,000 on average; 1:2600 in Turkey; 1:100,000 in Japan). Newborn screening, early diagnosis, and a synthetic (Phe-controlled) diet during infancy and childhood has alleviated the most severe outcomes in countries where testing and intervention is available. However, neonatal testing, first introduced mid-20th Century [8], is not yet universal; treatment is expensive and insurance coverage for medical foods remains problematic [9,10]. One outcome of successful dietary interventions is an increased population of socially integrated individuals living with PKU who are advocating for expanded non-dietary therapies. Many affected individuals struggle to obtain sufficient overall nutrition containing enough protein to satisfy essential metabolic needs while keeping Phe levels below the neurotoxic range. This problem is acute during pregnancy when high maternal Phe can cause irreversible fetal brain damage, regardless of whether the child will have PKU. Compounded by perpetual hunger, the high cost and inferior taste of most medical foods, and the basic human need for social inclusion, dietary compliance is problematic. Treatments allowing increased natural protein intake are desirable. An improved understanding of the molecular bases for PKU can help reach this goal.

The industry to address the unmet medical need of PKU includes about eight companies providing specialized foods/formulas, and one marketed therapeutic (Kuvan®), which is effective for some patients [11]. In addition, an injectable enzyme substitution therapy, Pegvaliase, is in clinical trials [12]. However, a recent survey of patients indicates strong desire for new, preferably oral, interventions that will reduce the need for dietary restrictions [13]. In short, there is a growing medical need, and a significant societal cost to not meeting this need. The consequences of untreated PKU has been estimated at a lifetime societal cost of >$1,000,000 per individual [12].

There is consensus that restoring the body's ability to maintain Phe in the normal range is key to effective therapy, although it is unresolved whether the best indicator of disease is blood Phe, brain Phe, or downstream neurotransmitter concentrations (e.g. [14,15]). Nevertheless, restoration of Phe regulation begins with understanding how unaffected individuals control Phe levels, and why there is extensive variation in phenotype among those who cannot regulate Phe. This article presents an updated framework for understanding human regulation of Phe, how this can go amiss, and ways of restoring affected individuals to a more normal Phe concentration range. The presented perspective is different from the common view; it is driven by, and focuses on, advances in knowledge of the structural biology of the enzyme phenylalanine hydroxylase (PAH; EC 1.14.16.1; OMIM 612349).

2. Regulation of Phe in humans

Phe in humans is regulated by PAH, which functions largely in the liver, and whose dysfunction is the cause of most PKU. An understanding of normal PAH structure/function relationships provides context for repairing abnormal PAH function. Dysfunctional PAH may have one or more incapacitated characteristics of normal PAH, while the other characteristics remain intact. PAH is able to convert Phe to another common amino acid, tyrosine, using molecular oxygen, tetrahydrobiopterin (BH_4) and a non-heme iron; this chemistry occurs at the PAH active site. PAH accomplishes the regulation of Phe through a structural flexibility that allows the protein to exist as a mixture of native structures that represent "off" and "on". In the "off" state (called resting-state PAH, (RS-PAH)), Phe cannot easily get into the active site. In the "on" state (called activated PAH (A-PAH)), the active site is fully accessible. RS-PAH and A-PAH exist as an equilibrating mixture of architecturally distinct assemblies, whose structures are used as symbols in Fig. 1. At low Phe (<~50 μM in normal individuals), the preferred PAH assembly is RS-PAH. RS-PAH is the molecular guard, watching and waiting, having a low affinity for Phe, and allowing a basal level of Phe to remain available for *essential* functions such as protein biosynthesis. As Phe levels rise, the PAH structural equilibrium shifts toward the activated assembly, which is A-PAH. A-PAH avidly binds Phe at the active site, converting it to tyrosine; this returns Phe levels to a concentration where RS-PAH again predominates. RS-PAH and A-PAH differ in the ability to convert Phe to tyrosine because they differ in their ability to productively bind Phe at the active site. However, the equilibrium between RS-PAH and A-PAH depends upon Phe binding to another site on the protein, called the allosteric site, which is formed by a structural change repositioning various parts of the PAH protein in the transition from RS-PAH to A-PAH. The precise structural location of this allosteric site, which Fig. 1 shows as present only in the A-PAH structure, was first hypothesized in 2013 [16]; it is now strongly supported by newly published structural studies [17–20]. In contrast, some earlier studies had generally suggested that allosteric Phe binding involved an intermolecular interaction involving the regulatory domain; these studies were interpreted solely on the basis of an RS-PAH structure model and did not foresee the conformational change required for formation of A-PAH (e.g. [21]).

The presented Phe-centric view of PAH function stresses the *regulation* of Phe concentration, not the production of tyrosine. The need for tyrosine does not drive PAH activity; the availability of Phe does. To regulate Phe, PAH must be able to interconvert between the RS-PAH and A-PAH assemblies in response to fluctuations in Phe. *PAH variants that cannot do this will likely be disease-associated*. Thus, PAH variants defective in the ability to properly modulate the PAH structure equilibrium (Fig. 1), expand our understanding of the repertoire of disease-associated PAH. The updated view of PKU does not dismiss the long-appreciated fact that a small number of disease-associated PAH variants 1) are truncated proteins that cannot fold and assemble properly or 2) are missing key active site components and cannot transform Phe to tyrosine, regardless of whether or not they fold, assemble, or convert between the RS-PAH and A-PAH structures. But the updated view departs from an oft-cited dogma that all other disease-associated PAH variants are defective in folding and/or stability. A portion of the >600 different disease-associated PAH variants have been shown to be prone to aggregation and/or degradation. These variants likely cannot properly form either the RS-PAH or the A-PAH structures. But an equally significant portion does not fall into this category. Unfortunately the concept of protein misfolding is often broadly applied in the absence of an appreciation for a metastable equilibrium of alternate protein structures, each of which is native, properly folded, and similar in energy (stability) to one another [22,23]. Here we abandon the concept of "misfolding with loss-of-function" and put a structural framework to prior proposals that disease-associated PAH variants can be defective in the allosteric response to elevated Phe in a manner that does not impair protein stability and/or promote aggregation (e.g. [21,24]). On the basis of newly available structural information that defines the allosteric Phe binding site, we propose that there is a class of common disease-associated variants that are not defective in the ability to fold, but are defective in the ability to stabilize A-PAH. Other variants are likely defective in the ability to transition from the "off" state to the "on" state.

3. Structure changes required for PAH to respond to allosteric Phe binding

This section describes what is known about the structures of RS-PAH and A-PAH and how this knowledge reshapes our understanding of the breadth of PAH variants that can contribute to PKU.

PAH is encoded by a single gene (location 12q23.2), which normally yields a 452 amino-acid long protein that folds and assembles