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Charakterisierung eines Mausmodells zur BH_4-sensitiven Phenylketonurie

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Ich erkläre hiermit an Eides statt, dass ich die vorliegende Dissertation mit dem Thema

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Anna Eichinger

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"Hope brings comfort. What has been need not forever continue to be so.
It is too late for some of our children, but if their plight can make people realize how unnecessary much of the tragedy is, their lives, thwarted as they are, will not have been meaningless."

"The Child Who Never Grew"
Pearl S. Buck; 1892-1973

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1 Einleitung

1.1 Phenylketonurie

1.1.1 Allgemeines

Die Phenylketonurie (PKU, OMIM # 261600) ist eine autosomal-rezessiv vererbte Erberkrankung, die auf Mutationen im *Phenylalaninhydroxylase*-Gen (*PAH*-Gen, ~ 98 %) bzw. im Synthese- und Regenerationsweg von Tetrahydrobiopterin (BH_4, ~ 2 %) beruht (Williams *et al.* 2008) (Abb. 1). Aufgrund der gestörten Hydroxylierung von Phenylalanin (L-Phe) zu Tyrosin (L-Tyr) kommt es zu einem Konzentrationsanstieg der essentiellen Aminosäure Phenylalanin und einiger Metaboliten (wie zum Beispiel Phenylpyruvat, Phenylacetat und Phenyllactat) im Blut und in weiteren Körperkompartimenten (Kaufman 1989).

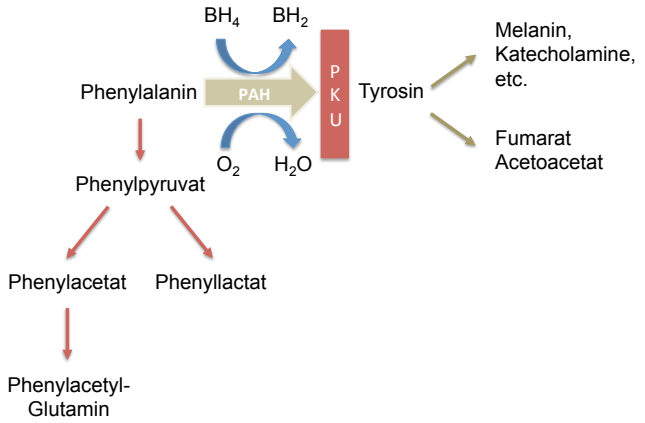

Abbildung 1. Phenylalanin-hydroxylase (PAH)-Reaktion. Bevorzugter Stoffwechsel (nach rechts verweisende Pfeile) sowie Entstehung von alternativen Metaboliten (nach unten weisende Pfeile) bei reduzierter PAH-Aktivität im Rahmen einer Phenylketonurie (PKU).

Die Klassifikation der Erkrankung erfolgt historisch anhand der Phenylalaninkonzentration im Plasma zum Zeitpunkt der Diagnosestellung und damit vor Beginn therapeutischer Maßnahmen: Klassische PKU mit [Phe] > 1 200 µmol/l (> 20 mg/dl), Milde PKU mit [Phe] 600 – 1 200 µmol/l (10 – 20 mg/dl) und Hyperphenylalaninämie (HPA) mit Phenylalaninkonzentrationen von 120 - 600 µmol/l (2 - 10 mg/dl) (Williams et al. 2008). Diese Gruppierung ist jedoch arbiträr und folgt keiner biologischen Grundlage. Vielmehr handelt es sich um ein Kontinuum an residualer Enzymaktivität, wobei eine inverse Korrelation zwischen PAH-Enzymaktivität und Phenylalanin-konzentration besteht. Inzwischen besteht Konsens darüber, dass unbehandelte Patienten mit Phenylalaninkonzentrationen von weniger als 360 µmol/l keine Therapie benötigen. Patienten mit höheren Werten werden diätetisch oder medikamentös behandelt (van Spronsen *et al.* 2017).
Die PKU ist mit einer Inzidenz von 1 bis 5 zu 10 000 Lebendgeburten die häufigste angeborene Aminosäure-Stoffwechselstörung in Europa (Steinfeld *et al.* 2004; Scriver 2006), wobei deutliche Unterschiede zwischen verschiedenen Bevölkerungsgruppen vorliegen (Hardelid *et al.* 2008; Williams *et al.* 2008; Al Hafid and Christodoulou 2015). In Deutschland lag die Prävalenz in den Jahren 2004 bis 2012 bei 1 zu 5 316 (Gramer 2015). Ohne adäquate Therapie erleiden die Patienten eine schwere neuro-psychologische Schädigung, so dass ein eigenständig geführtes Leben zumeist unmöglich ist (Murphy *et al.* 2008) und die Lebenserwartung reduziert ist (Jancar 1998). Der aktuelle therapeutische Standard besteht in der Durchführung einer speziellen phenylalaninarmen Diät (nicht BH_4-sensitive Patienten bzw. nicht ausreichend BH_4-sensitive Patienten) und/oder einer Therapie mit dem

Medikament Sapropterin-Dihydrochlorid (Kuvan®; Patienten mit BH_4-sensitiver Erkrankung oder BH_4-Metabolismus-Defekt)(van Spronsen *et al.* 2017).
Die Erstbeschreibung der Erkrankung erfolgte im Jahr 1934 durch Dr. Asbjørn Følling als „*oligophrenia phenylpyruvica*"(Følling 1934), eine Stoffwechselanomalie, die mit Ausscheidung von Phenylbrenztraubensäure im Urin und mit mentaler Retardierung einhergeht. Bereits in den 1950er Jahren entwickelte der deutsche Kinderarzt Horst Bickel mit einer phenylalaninarmen Diät die erste wirksame Behandlung der Erkrankung (Bickel *et al.* 1953). Mithilfe der Entwicklung eines Screening-Tests für die PKU durch den Biochemiker Bob Guthrie gab es seit den 1960er Jahren eine flächenweit anwendbare Methode (Guthrie and Susi 1963; Guthrie 1969), um die Erkrankung frühzeitig zu erkennen und anschließend effektiv behandeln zu können (Abb. 2).

Abbildung 2. Arvada's Poster Girls – *„There is hope for the mentally retarded."*
1961 wählte die NARC (*National Association for Retarded Children*) zwei Schwestern mit PKU als *Poster Girls* für ihre jährliche Publikation aus (www.arjc.org). Bei Sheila McGrath (6 Jahre; rechts), der älteren Schwester, wurde die Erkrankung nicht rechtzeitig diagnostiziert, aber ihre jüngere Schwester Kammy (4 Jahre; links) konnte sich dank der frühen Diagnose und rechtzeitigen Behandlung normal entwickeln (Koch 2006).

1.1.2 Symptomatik

Die reduzierte PAH-Aktivität führt zu einer Hyperphenylalaninämie sowie zu einem Mangel an L-Tyrosin und nachfolgenden Stoffwechselprodukten (siehe 1.2.3). Unbehandelte Patienten haben auf Grund des aus der Tyrosinreduktion resultierenden Melaninmangels im Vergleich zu gesunden Familienmitgliedern hellere Haut, Haare und Augen (Farishian and Whittaker 1980). Sie entwickeln häufig ekzematöse Ausschläge (Knapp 1964) sowie sklerodermiforme Hautveränderungen (Nova *et al.* 1992). Die Haupterkrankungslast besteht jedoch in der mentalen Retardierung, die bei unbehandelten Patienten in unterschiedlichen Schweregraden auftritt (Scriver 2001). Es zeigen sich eine allgemeine Entwicklungsverzögerung mit einem verspäteten bzw. einem Nichterreichen der Meilensteine der Entwicklung sowie eine zum Teil gravierende Verminderung des Intelligenzquotienten (Waisbren *et al.* 2007). Je nach Ausmaß der Schädigung finden sich Verhaltensauffälligkeiten (wie aggressive sowie autoaggressive Tendenzen), krankhafte Bewegungsmuster (wie Spastik und Tremor) sowie epileptische Anfälle (Paine 1957). Zerebrale MRT-Untersuchungen zeigen häufig periventrikuläre und frontale Demyelinisierungen (Anderson and Leuzzi 2010; Mastrangelo *et al.* 2015) sowie atrophe Veränderungen (Karimzadeh *et al.* 2014), die zum Teil mit dem Ausmaß der Therapieadhärenz korrelieren (Mastrangelo *et al.* 2015).
Patientinnen mit Hyperphenylalaninämie müssen frühzeitig und konsequent zum Phänomen der maternalen Phenylketonurie aufgeklärt werden. Hierbei handelt es sich um eine

phenylalanininduzierte Fetopathie, die bereits bei geringgradigen Erhöhungen der mütterlichen Phenylalaninkonzentration (> 360 µmol/l) zur Schädigung des ungeborenen Kindes führt. Die charakteristischen kindlichen Symptome sind ein intrauteriner Wachstumsrückstand, Mikrozephalie mit kognitiver Beeinträchtigung sowie Herzvitien (Koch 2008; Prick *et al.* 2012). Patientinnen mit Kinderwunsch bedürfen daher einer besonders intensiven Betreuung präkonzeptionell (Commitee 2015) und während der gesamten Schwangerschaft (Longo *et al.* 2015; van Spronsen *et al.* 2017).
Aufgrund der deutlich verbesserten Behandlungsmöglichkeiten der Patienten und des ständigen Zugewinns an Wissen und damit an therapeutischen Optionen sollte eine kontinuierliche, lebenslange Anbindung aller Patienten an spezialisierte Stoffwechselzentren angestrebt werden (Nagasaka *et al.* 2014).

1.1.3 Therapeutische Möglichkeiten

Die klassische Behandlung der PKU besteht in der Durchführung einer streng phenylalaninarmen Diät. Die Menge an natürlichem Protein ist so gering, dass zusätzlich eine Proteinsubstitution mit Hilfe von phenylalaninfreien Aminosäuremischungen erforderlich ist, die zusätzlich mit Vitaminen, Spurenelementen und Mineralstoffen angereichert ist. Die Behandlung sollte so früh wie möglich beginnen (Koch *et al.* 2002; Bosch *et al.* 2007) und lebenslänglich fortgeführt werden (Lou *et al.* 1985; Rey *et al.* 1996; Cerone *et al.* 1999; Channon *et al.* 2007; Vockley *et al.* 2014; van Spronsen *et al.* 2017). Auch bei einer späten Diagnose profitieren die Patienten von einer Therapieeinleitung (Grosse 2010). Neben den Phenylalaninkonzentrationen im Blut sollten auch die anderen Aminosäuren und Mikronährstoffe sowie klinische Parameter wie die Gewichts- und Wachstumsperzentilen und die psychomotorische Entwicklung engmaschig überwacht werden.
Die sehr rigide Therapie führt zu exzellenten klinischen Erfolgen, bringt aber erhebliche Belastungen für die Patienten und ihre Familien mit sich. Unter anderem kann es zu Mangelernährung (Gropper *et al.* 1988; Reilly *et al.* 1990; Zeman *et al.* 1999), Einschränkungen im sozialen Lebensbereich (Weglage *et al.* 1996) sowie psychischen Auffälligkeiten (Manti *et al.* 2016) kommen. Auf Dauer tritt daher häufig eine unzureichende Adhärenz in Bezug auf die strenge Diät auf, was schwere gesundheitliche Folgeschäden hervorrufen kann (Walter *et al.* 2002; Didycz and Bik-Multanowski 2018). Aus diesem Grund und weil auch unter exakt durchgeführter diätetischer Therapie diskrete neurologische Defizite zu finden sind (Burgard *et al.* 1996; Palermo *et al.* 2017), bemühen sich Ärzte und Wissenschaftler um die Entwicklung alternativer Behandlungsmöglichkeiten.

Vor einigen Jahren wurde eine neue Therapieform etabliert. Es wurde zunächst an einzelnen Patienten gezeigt, dass die orale Gabe von BH_4, dem natürlichen Kofaktor der PAH, auch bei Vorliegen von Mutationen im *PAH*-Gen ohne absoluten BH_4-Mangel die Phenylalaninkonzentration reduzieren kann (Kure *et al.* 1999; Muntau *et al.* 2002). Später wurde in einer ersten systematischen Studie gezeigt, dass pharmakologische Dosen von BH_4 bei einem signifikanten Prozentsatz von Patienten mit residualer Enzymaktivität die Phenylalaninkonzentration im Blut senken sowie die PAH-Enzymaktivität *in vivo* und dadurch die diätetische Phenylalanin-Toleranz erhöhen (Muntau *et al.* 2002). BH_4-sensitive Patienten können durch die Durchführung eines oralen BH_4-Belastungstests identifiziert werden. 2008 wurde Sapropterin-Dihydrochlorid (Kuvan®), die synthetische Form von BH_4, als *Orphan Drug* von der EMA zur alleinigen oder supplementären Therapie der BH_4-sensitiven PKU zugelassen. In klinischen Studien wurden gute klinische Erfolge ohne relevante Nebenwirkungen nachgewiesen (Levy *et al.* 2007; Trefz *et al.* 2009; Longo *et al.* 2015; Trefz *et al.* 2015; Muntau *et al.* 2017).
Die diätetische Einstellung in der Schwangerschaft ist häufig sehr schwierig und wird durch die oft bestehende Hyperemesis weiter erschwert. Besonders Patientinnen, die aufgrund ihrer milden

Erkrankung nur in der Schwangerschaft einer spezifischen Therapie bedürfen, sind hiervon betroffen. Die Medikation mit Kuvan® wird in diesen Fällen häufig gut toleriert und bisher zeigt sich eine gute Wirksamkeit der BH_4-Therapie bei unauffälligen fetalem Sicherheitsprofil (Feillet *et al.* 2014; Trefz *et al.* 2015; van Spronsen *et al.* 2017).

Ein weiterer therapeutischer Ansatz beinhaltet die Supplementation von großen neutralen Aminosäuren (LNAA, wie Valin, Isoleucin und Leucin), um die Aufnahme von Phenylalanin in das Gehirn zu reduzieren (Matalon *et al.* 2007; Yano *et al.* 2016). Die Ergebnisse werden kontrovers diskutiert und ein langfristiger klinischer Erfolg konnte bisher nicht sicher nachgewiesen werden. Wesentlich vielversprechender ist die subkutane Verabreichung des pegylierten bakteriellen Enzyms Phenylalanin-Ammoniak-Lyase, das Phenylalanin zu *trans*-Zimtsäure und geringen Mengen Ammoniak umsetzt (Sarkissian *et al.* 1999). Die Substanz hat inzwischen alle klinischen Studienphasen durchlaufen und die Zulassung als *Orphan Drug* mit dem Namen Pegvaliase® wurde in den USA (FDA) beantragt. Die Wirksamkeit für Patienten aller Schweregrade wird als sehr gut bewertet; es treten jedoch zum Teil erhebliche immunologisch ausgelöste Nebenwirkungen auf (Harding *et al.* 2018; Thomas *et al.* 2018). Die internationale europäische Studie unter Leitung der Arbeitsgruppe *Muntau/Gersting* wird in Kürze initiiert.
Darüber hinaus gibt es verschiedene Forschungsansätze im Bereich der Gentherapie (Yagi *et al.* 2011), wobei mit verschiedensten Vektoren gearbeitet wird, die eine stabile Expression des *PAH*-Gens erreichen sollen. Hiermit soll die anhaltende Synthese einer normal funktionsfähigen PAH gewährleistet werden (Yagi *et al.* 2011; Viecelli *et al.* 2014). In diesem Bereich gibt es nun auch bereits erste *in vitro* Daten mit der Anwendung der neuen CRISPR/Cas9-basierten Methodik (Pan *et al.* 2016).

1.2 Humane Phenylalaninhydroxylase

1.2.1 *PAH*-Gen und Mutationen

Das humane *PAH*-Gen (*Hs PAH*, Entrez Gene 5053) ist auf dem Chromosom 12 q22-24.1 lokalisiert und erstreckt sich über ~ 90 kb genomische DNA (GenBank accession no. NG_008690.1). Das Gen enthält 13 Exons und die zugehörige cDNA hat eine Länge von ca. 2 500 bp (GenBank accession no. NM000277, cDNA) (Scriver *et al.* 2003).
In der 5' untranslatierten Region (~25 kb) finden sich verschiedene regulatorische Elemente, die Kennzeichen typischer *Housekeeping*-Gen-Steuerung aufweisen. Es konnten hierbei GC-reiche Domänen sowie eine kanonische CCAAT-Sequenz identifiziert werden. Darüber hinaus wurden zwei Regionen identifiziert, die Glucokorticoid-responsiven Elementen ähneln und möglicherweise einen Beitrag zur PAH-Aktivitäts-Regulation leisten. Es wurden keine typischen *cis-acting* Elemente identifiziert, die mit Leber-spezifischen Regulationsmechanismen in Zusammenhang gebracht werden (Konecki *et al.* 1992).
Mittlerweile wurden über 750 *PAH*-Mutationen beschrieben (hgmd.org) (Human Gene Mutation Database: 762 Mutationen am 02.09.2018). Ein Großteil davon (etwa 65 %) sind *Missense*-Mutationen, d.h. Mutationen, die zu dem Austausch einer einzelnen Aminosäure führen (Waters *et al.* 1999; Waters *et al.* 2000; Gersting *et al.* 2008). Dies kann verschiedene Folgen haben, wie z.B. den Funktionsverlust der PAH (v.a. bei Mutationen im aktiven Zentrum), Veränderungen der enzymkinetischen Parameter und/oder der Stabilität des Proteins, was zu vermehrter Aggregation und Degradation führen kann (Gregersen *et al.* 2000; Gersting *et al.* 2008). Des Weiteren kommen Deletionen, Insertionen, *Splice*- und *Stop*-Mutationen vor.

Bei den meisten Patienten liegt eine *Compound*-Heterozygotie vor, d.h., sie tragen auf jedem der beiden Allele eine unterschiedliche Mutation. Dies trägt in erheblicher Weise zur genetischen Heterogenität bei und erschwert erheblich Aussagen über den Phänotyp auf der Basis des Genotyps (Okano *et al.* 1991; Guldberg *et al.* 1998; Leandro *et al.* 2011; Djordjevic *et al.* 2013; Shen *et al.* 2016). Unsere Arbeitsgruppe hat einen zellbasierten *Assay* entwickelt, der bei Expression von zwei *PAH*-Mutationen den Genotyp von Patienten nachbilden kann (Staudigl *et al.* 2011). Die Messung der PAH-Enzymaktivität bei verschiedenen Kombinationen von L-Phe- und BH_4-Konzentrationen ermöglicht es, das Arbeitsoptimum des varianten Proteins des Patienten im metabolischen (L-Phe) und therapeutischen (BH_4) Kontext zu bestimmen. Nach bioinformatischer Auswertung und Anwendung einer Farbkodierung werden die Ergebnisse als sogenannte PAH *Activity Landscapes* dargestellt. Eine Webapplikation eröffnet Kollegen weltweit Zugang zu den Daten, die im Sinne der personalisierten Medizin in der Patientenbetreuung Anwendung finden, um die diätetische oder pharmakologische Therapie zu optimieren (Danecka *et al.* 2015; pah-activitylandscapes.org 2018).

1.2.2 Die Phenylalaninhydroxylase

Die PAH (Phenylalanin-4-Monooxygenase; EC 1.14.16.1) hat eine Länge von 452 Aminosäuren (AS) und setzt sich aus drei Domänen mit unterschiedlichen Funktionen zusammen (Arturo *et al.* 2016). Die N-terminale regulatorische Domäne (AS 1 – 117 der hs PAH) beinhaltet eine autoregulative Sequenz (AS 1 – 30) mit einer Phosphorylierungsstelle (Ser16), die für regulatorische Prozesse von Bedeutung ist. Die wichtigsten aktivierenden Prozesse sind die PKA-abhängige Ser16-Phosphorylierung sowie die positive Kooperativität, die durch L-Phe-Inkubation hervorgerufen wird und vom aktiven Zentrum unabhängig ist (Miranda *et al.* 2002; Roberts *et al.* 2014). Die Bindung des Kofaktors BH_4 hat einen allosterischen inhibitorischen Effekt (Flydal and Martinez 2013). Die katalytische Domäne (AS 118 – 410) enthält das aktive Zentrum mit dem Eisenatom, der L-Phe- und der BH_4-Bindungsstelle. Die C-terminal gelegene Oligomerisierungs-Domäne (AS 411 – 452) besteht aus einem anti-parallelen β-sheet (AS 411 – 424) als Dimerisierungsmotiv und einem *coiled-coil*-Motiv (AS 428 – 452), das durch antiparallele Helix-Helix-Interaktionen zur Oligomerisierung beiträgt (Abb. 3, links).
Zytosolisch liegt das Enzym als Tetramer vor, das sich als „Dimer zweier Dimeren" darstellt (Abb. 3, rechts). Die Untereinheiten der PAH sind strukturell eng miteinander verbunden und arbeiten in den katalytischen und vor allem regulatorischen Prozessen zusammen.
Hierbei gibt es unterschiedliche Mechanismen, die zusätzlich auf die Aktivität des Enzyms Einfluss nehmen. Die Phosphorylierung an Ser16, die unter anderem von verschiedenen Hormonen und der Plasma-Phenylalaninkonzentration mitbeeinflusst wird (Kaufman 1993), führt zu einem Anstieg der basalen PAH-Aktivität. Diese wird auch über Konformationsänderungen, die durch die Bindung des Substrates L-Phe und des Kofaktors BH_4 hervorgerufen werden, beeinflusst. Die Anwesenheit von L-Phe führt zu einem Anstieg der PAH-Aktivität im Sinne einer positiven Kooperativität (Stokka *et al.* 2004), sodass bei steigender Serum-Phenylalaninkonzentration die PAH aktiviert wird (Thórólfsson *et al.* 2003). Der Kofaktor BH_4 hingegen wird nicht nur in der direkten PAH-Reaktion benötigt, sondern wirkt auch als allosterischer Inhibitor. In dieser Funktion führt BH_4 dazu, dass das Enzym bei niedrigen Phenylalaninkonzentrationen in einem Zustand geringerer Aktivität verweilt. Hierbei wird das Protein stabilisiert, kann jedoch bei Bedarf (Anstieg der Phenylalaninkonzentration) schnell wieder in ein höheres Aktivitätsniveau rücküberführt werden (Mitnaul and Shiman 1995).

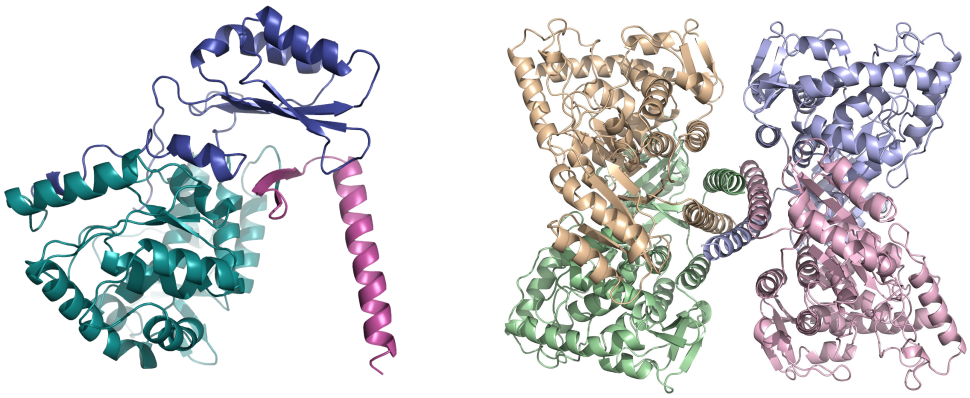

Abbildung 3. PAH-Monomer (links) und PAH-Tetramer (rechts).
Das Monomer (links) setzt sich aus drei Domänen zusammen, der N-terminal lokalisierten regulatorischen (dunkelblau), der katalytischen (grün) und der C-terminalen Oligomerisierungsdomäne (magenta). Funktionell liegt die PAH als Tetramer (rechts) vor (gelb/hellgrün, sowie helles magenta/hellblau).
Unveröffentlichte Daten der Arbeitsgruppe *Muntau/Gersting*.

1.2.3 Funktion der Phenylalaninhydroxylase

Die Phenylalaninhydroxylase ist das Schlüsselenzym im Phenylalanin-Metabolismus (Abb. 4). Sie katalysiert die *para*-Hydroxylierung der essentiellen Aminosäure L-Phenylalanin zu L-Tyrosin. Dafür ist die Anwesenheit des Kofaktors (6R)-L-*erythro*-5,6,7,8-Tetrahydrobiopterin (BH_4), von Eisen und von molekularem Sauerstoff (O_2) erforderlich (Scriver 2001).
Die Leber stellt den Hauptanteil der intrazellulären PAH zur Verfügung und ist somit das wichtigste Organ für die Aufrechterhaltung des Phenylalanin-Stoffwechsels. Beim Menschen weisen Niere, Gehirn, Pankreas und die Melanozyten ebenfalls eine geringe PAH-Aktivität auf (Lichter-Konecki *et al.* 1999).
Tyrosin wird im Weiteren für die Produktion verschiedener Proteine benötigt. Unter anderem kommt es bei erniedrigter Tyrosinkonzentration in Kombination mit erhöhten Phenylalaninwerten zu einer unzureichenden Synthese von Melanin, was zu dem charakteristischen Haut- und Haarerscheinungsbild von PKU-Patienten führt (Farishian and Whittaker 1980). Die genaue Pathogenese der neurologischen Schädigung ist bisher nicht aufgeklärt, aber man geht davon aus, dass sie durch eine Kombination unterschiedlicher Mechanismen entsteht (de Groot *et al.* 2010; Schuck *et al.* 2015). L-Phenylalanin konkurriert mit anderen Aminosäuren um den Transport in das Gehirn. Die Menge an frei verfügbarem L-Tyrosin als Ausgangspunkt für die Synthese der Neurotransmitter und Katecholamine Dopamin, Noradrenalin und Adrenalin ist verändert. Im Gehirn zeigen sich veränderte DNA-Methylierungs- (Dobrowolski *et al.* 2016) und Protein-Expressionsmuster (Imperlini *et al.* 2014), Beeinträchtigungen des Neurotransmitter-Haushalts (Kienzle Hagen *et al.* 2002) und veränderte Rezeptor-Funktionen (Glushakov *et al.* 2002). Die Patienten weisen Zeichen von vermehrtem oxidativem Stress auf (Schulpis *et al.* 2005) und zeigen Veränderungen im Lipid-Stoffwechsel (Nagasaka *et al.* 2014).

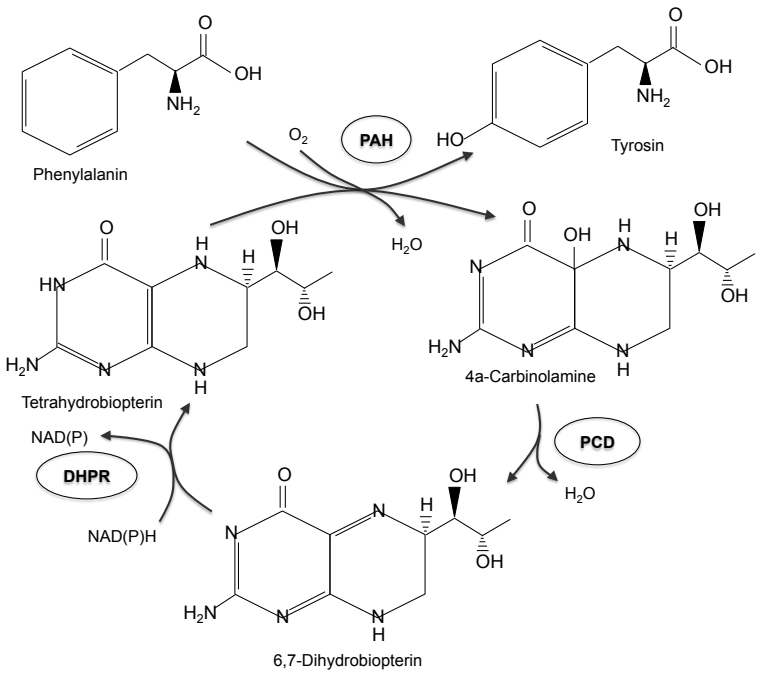

Abbildung 4.
Das Phenylalanin-Hydroxylierungs-System.
In Anwesenheit des natürlichen Kofaktors BH_4 katalysiert die Phenylalaninhydroxylase (PAH) die Hydroxylierung von L-Phe zu L-Tyr. BH_4 wird dabei über 4a-Hydroxy-Tetrahydrobiopterin zu 6,7-Dihydrobiopterin oxidiert und mit Hilfe der NADH/H^+ abhängigen DHPR (Dihydropteridin-Reduktase) wieder zu BH_4 reduziert. PCD = Pterin-4a-Carbinolamin-Dehydratase. Modifiziert nach (Pribat et al. 2010).

Die Familie der Aromatischen-Aminosäure-Hydroxylasen (AAH) umfasst neben der PAH auch die Tyrosin- und die Tryptophanhydroxylase. Diese drei Enzyme sind strukturell und funktionell eng verwandt und benötigen alle ein Eisen-Atom und BH_4, um eine Hydroxy-Gruppe in den aromatischen Ring einbringen zu können. Dabei ist BH_4 der Elektronen-Donor für die Reduktion von Fe^{3+} und die Bildung von Wasser (H_2O) unter O_2-Verbrauch. Der Kofaktor BH_4 wird dabei über 4a-Hydroxy-Tetrahydrobiopterin zur quinoiden Form des 6,7-Dihydrobiopterin (BH_2) oxidiert. Die Regeneration zu BH_4 erfolgt mit Hilfe der Dihydropteridin-Reduktase (DHPR) (Pey *et al.* 2006). Des Weiteren kann BH_4 auch aus Guanosintriphosphat (GTP) hergestellt werden (Abb. 5).

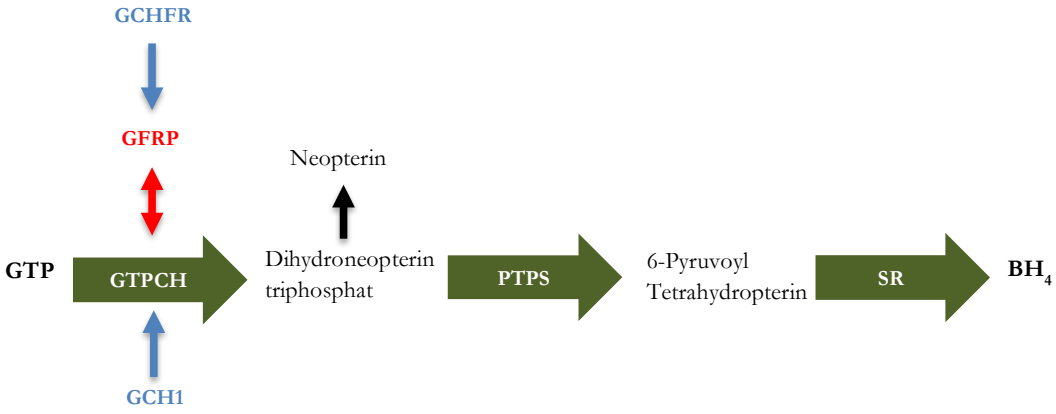

Abbildung 5. *De novo*-Synthese von Tetrahydrobiopterin (BH_4).
Die *De-novo*-Synthese von BH_4 erfolgt über 7,8-Dihydroneopterin-Triphosphat und 6-Pyruvoyl-5,6,7,8-Tetrahydrobiopterin. Die Reaktionen werden von den Enzymen GTP-Cyclohydrolase (GTPCH, kodiert vom *GCH1* Gen), von der 6-Pyruvoyl-5,6,7,8-Tetrahydropterin-Syntase (PTPS) und der Sepiapterin-Reduktase (SR) katalysiert. Das geschwindigkeitsbestimmende Enzym der Synthese ist die GTPCH, welche durch das GTP Cyclohydrolase Feedback Regulatory Protein (GFRP, kodiert vom *GCHFR* Gen) reguliert wird, welches durch BH_4 gehemmt und durch L-Phe stimuliert wird. Modifiziert nach (Tatham *et al.* 2009).

1.3 PKU als Proteinfaltungserkrankung und der BH_4-Effekt

Bereits seit längerem ist bekannt, dass einer der wichtigsten Pathomechanismen bei *Missense*-Mutationen die veränderte Faltung des Proteins ist, die zu beeinträchtigter Proteinkonformation führt (Waters *et al.* 1998). Mittlerweile gilt die PKU als eines der Paradebeispiele für eine Proteinfaltungserkrankung mit *„Loss of Function"* (Muntau and Gersting 2010). Strukturelle Veränderungen des Enzyms können zu Veränderungen der Oligomerisierung, vermehrter Aggregation und reduzierter Proteinstabilität mit Beeinträchtigung der enzymkinetischen Parameter führen (Pey *et al.* 2007; Gersting *et al.* 2008). Der Austausch einzelner Aminosäuren innerhalb der PAH kann darüber hinaus spezifische Inter- und Intradomänen-Wechselwirkungen beeinträchtigen, die für die konformationelle Stabilität und Flexibilität des Proteins verantwortlich sind (Gersting *et al.* 2008).

Früh gab es Hinweise darauf, dass der pharmakologische Effekt von BH_4 (siehe 1.1.3) über eine Korrektur der Proteinfehlfaltung und eine Zunahme der Proteinstabilität vermittelt werden könnte (Pey *et al.* 2004; Perez *et al.* 2005). Pharmakologische Dosen von BH_4 führen bei BH_4-sensitiven Patienten (ca. 30 % aller PKU-Patienten) zu einer Zunahme der PAH-Aktivität *in vivo*, obwohl bei den Patienten kein absoluter BH_4-Mangel vorliegt (Muntau *et al.* 2002). Die meisten der BH_4-sensitiven Patienten haben einen milden bzw. moderaten Phänotyp und weisen keine Varianten auf, die zu einem totalen Aktivitätsverlust der PAH führen (Gersting *et al.* 2008; Zurflüh *et al.* 2008).

Es konnte gezeigt werden, dass BH_4 als pharmakologischer Chaperon wirkt, indem es an das fehlgefaltete PAH-Protein bindet, den Faltungsprozess positiv beeinflusst und damit die Faltungsdynamik hin zur richtigen Konformation verschiebt, so dass insgesamt mehr korrekt gefaltete PAH vorliegt (Blau and Erlandsen 2004; Muntau and Gersting 2010). Darüber hinaus wurde kürzlich eine Defizienz von DNAJC12 (DNAJ/HSP40 homolog, *subfamily C, member 12*) beschrieben (Anikster *et al.* 2017). Bei Erniedrigung oder Fehlfunktion dieses Proteins, das als Co-Chaperon mit für die korrekte Faltung der PAH, der Tyrosin- und der Tryptophanhydroxylase zuständig ist, kommt es ebenso zu einer BH_4-sensitiven Hyperphenylalaninämie und zu einem Neurotransmittermangel (Blau *et al.* 2018).

Derzeit wird nach weiteren pharmakologischen Chaperonen gesucht, die sich zur Behandlung von nicht BH_4-sensitiven Patienten eignen (Pey *et al.* 2008; Santos-Sierra *et al.* 2012; Hole *et al.* 2016).

1.4 Mausmodell der Phenylketonurie

1.4.1 Die murine Phenylalaninhydroxylase

Das murine *Phenylalaninhydroxylase*-Gen (*Mm Pah*, NC_000076.6) ist auf Chromosom 10 lokalisiert (Ledley *et al.* 1988) und die zugehörige mRNA hat eine Länge von ~ 2000 bp (GenBank accession no NM_008777.3). Das murine *Pah*-Gen hat eine Sequenzhomologie von 87 % zum humanen *PAH*-Gen, wobei es sich bei ca. 5 % um Basenunterschiede ohne Veränderung der Aminosäuresequenz handelt. In den untranslatierten Regionen werden keine auffälligen Homologien beobachtet.

Das Protein Mm Pah besteht aus 453 AS und hat ein molekulares Gewicht von 52 786 Da. Dabei zeigt sich eine 92 %-ige Homologie zwischen der humanen und der murinen Aminosäure-Folge (Abb. 6). Ein Großteil der Unterschiede ist dabei in der N-terminalen Region zu finden, dem Abschnitt, der eher eine regulatorische als eine katalytische Funktion hat.

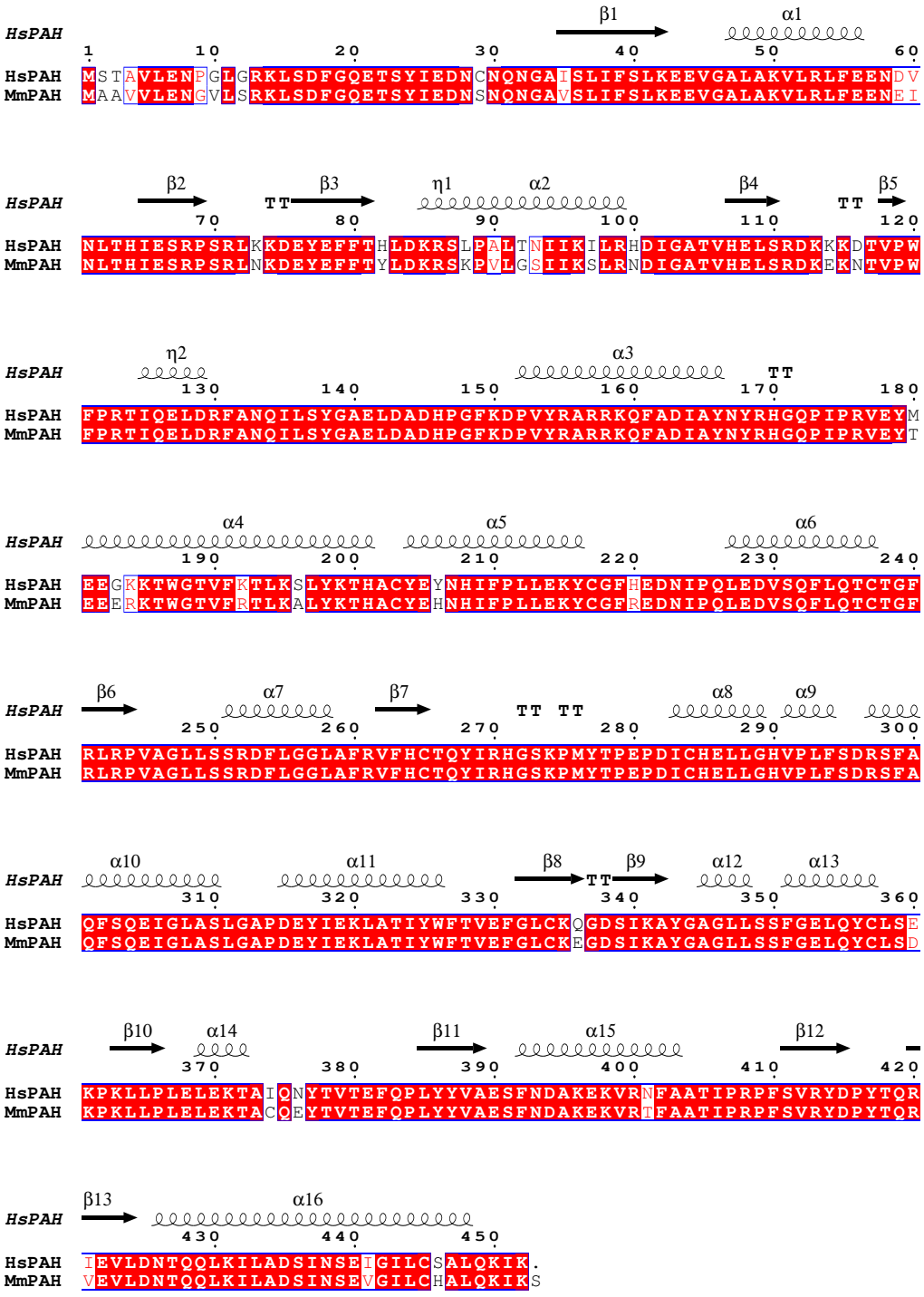

Abbildung 6. Vergleich der Aminosäure-Sequenzen der PAH: Mensch (hs PAH) und Maus (Mm Pah).
Unterschiede 34/453 = 7,5% (siehe auch Tbl. 1)
(Robert and Gouet 2014).

1.4.2 Beschreibung der einzelnen Mausmodelle der Phenylketonurie

1.4.2.1 Allgemeines

Nachdem lange Zeit Tiermodelle mit chemisch induzierten erhöhten Serum-Phenylalaninkonzentrationen verwendet wurden (Scriver 1989), konnten ab 1990 mit Hilfe des Mutagens Ethylnitrosourea (ENU) verschiedene Mausmodelle für die Phenylketonurie entwickelt werden (Shedlovsky *et al.* 1993). Im Weiteren wird genauer auf die Mauslinien *Pah*$^{enu1/enu1}$ (hph-5.0) (McDonald *et al.* 1990), *Pah*$^{enu2/enu2}$ (hph-5.1) und die *compound*-heterozygote *Pah*$^{enu1/enu2}$ (Sarkissian *et al.* 2000) eingegangen (Abb. 7). Die genetischen Veränderungen wurden für diese Tiere jeweils ausgehend von der Wildtyp-Linie BTBR (*Pah*$^{wt/wt}$, WT) eingeführt.

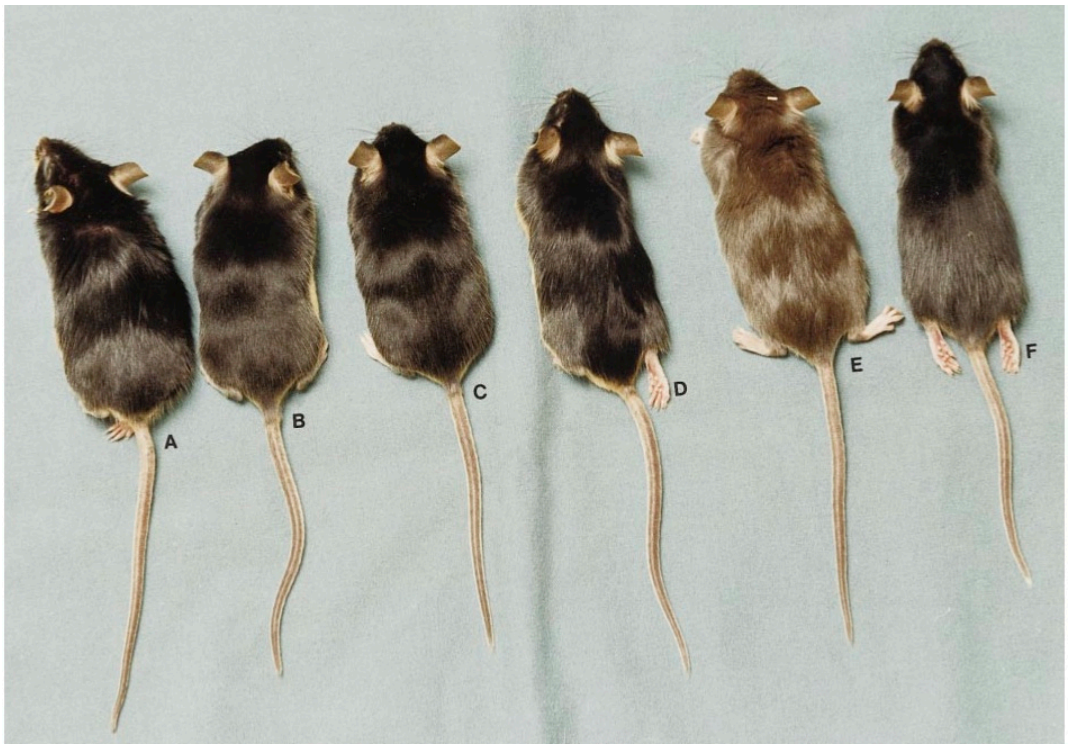

Abbildung 7. PKU- und HPA-Mausmodelle.
(A) *Pah*$^{wt/wt}$ (Wildtyp, BTBR), (B) *Pah*$^{wt/enu1}$, (C) *Pah*$^{enu1/enu1}$, (D) *Pah*$^{wt/enu2}$, (E) *Pah*$^{enu2/enu2}$, (F) *Pah*$^{enu1/enu2}$ (Sarkissian *et al.* 2000).

Die verschiedenen Mausmodelle werden verwendet, um die Pathophysiologie der neurologischen Schädigung und die klinischen Auswirkungen der Erkrankung, zum Beispiel im Rahmen des maternalen PKU-Syndroms, besser zu verstehen (McDonald *et al.* 1997; Yannicelli and Medeiros 2002). Außerdem dienen sie der Forschung zu innovativen Behandlungsmöglichkeiten, wie dem Einsatz der Phenylalanin-Ammoniak-Lyase (Liu *et al.* 2002; Sarkissian *et al.* 2011; Rossi *et al.* 2014; Pascucci *et al.* 2018), der Gentherapie (Nagasaki *et al.* 1999; Yagi *et al.* 2012), der Untersuchung des Wirkmechanismus von BH_4 (Gersting *et al.* 2010; Lagler *et al.* 2010; Sarkissian *et al.* 2012) und der pharmakologischen Stabilisierung der PAH durch chemische Moleküle (sog. *small molecules*) (Santos-Sierra *et al.* 2012).

1.4.2.2 Das Mausmodell *Pah*$^{enu1/enu1}$ (ENU1)

Pah$^{enu1/enu1}$ (ENU1; [p.Val106Ala/p.Val106Ala]) ist ein Modell für die Hyperphenylalaninämie und wird durch die *Missense*-Mutation p.Val106Ala (c.364T>C Transition; ehemalige Nomenklatur: V106A) hervorgerufen (Abb. 8).
Valin 106 ist unter den Säugetierspezies hoch konserviert, allerdings gibt es Unterschiede zu anderen Spezies. Die Mutation p.Val106Ala führt zu einem Aminosäure-Austausch im N-terminalen Bereich des Proteins, in dem die murine und die humane Sequenz die größten Unterschiede aufweisen (McDonald and Charlton 1997). Bei Patienten, die die Mutation p.Val106Ala tragen, findet sich eine milde PKU; Daten zur BH_4-Sensivität beim Menschen sind bisher nicht veröffentlicht (Okano *et al.* 2011). Die bekannten, in der näheren Umgebung liegenden Mutationen (p.Ala104Asp, p.Ser110Leu) sind mit einem milden, BH_4-sensitiven Phänotyp assoziiert. Sie führen zu einer Störung der Proteinkonformation und -stabilität (Kayaalp *et al.* 1997; Erlandsen and Stevens 1999).
Die homozygote *Pah*$^{enu1/enu1}$ zeigt unter normaler Ernährung einen milden Phänotyp mit normaler Fellfarbe und unauffälligen Ergebnissen in Verhaltens- und Intelligenztests (Abb. 9A). Allerdings führt eine erhöhte L-Phenylalanin-Zufuhr zu einer Wachstumsretardierung (McDonald *et al.* 1990). Die PAH-Enzymaktivität in der Leber ist erniedrigt (~ 10 – 25 % des Wildtyps) und die Phenylalaninkonzentrationen im Plasma sind nur leicht erhöht (Shedlovsky *et al.* 1993; Sarkissian *et al.* 2000; Gersting *et al.* 2010). Wie für einen heterozygoten Träger bei autosomal-rezessivem Erbgang zu erwarten, ist *Pah*$^{wt/enu1}$ phänotypisch normal und zeigt nur eine leicht erniedrigte Pah-Aktivität im Lebergewebe (Sarkissian *et al.* 2000).

1.4.2.3 Das Mausmodell *Pah*$^{enu2/enu2}$ (ENU2)

Pah$^{enu2/enu2}$ (ENU2; [p.Phe263Ser/p.Phe263Ser]) ist ein Modell für die klassische PKU und wird durch die Mutation p.Phe263Ser (c.835T>C Transition; ehemalige Nomenklatur F263S) in Exon 7 hervorgerufen (McDonald and Charlton 1997). Es kommt hierbei zum Austausch der aromatischen, unpolaren Aminosäure Phenylalanin zur polaren, nicht-ionisierbaren Aminosäure Serin in der katalytischen Domäne des Enzyms (Abb. 8), nahe der Eisen- und Pterin-Bindungsstelle (Erlandsen and Stevens 1999; Flatmark and Stevens 1999; Teigen *et al.* 1999; Erlandsen *et al.* 2000). Diese Mutation führt zu einem schweren Phänotyp. Beim Menschen wurde an der Position Phe 263 die Mutation p.Phe263Ser sowie die Mutation p.Phe263Leu (ehemalige Nomenklatur F263L) beschrieben, die zu einem fast vollständigen Verlust der PAH-Aktivität und zu einem schweren, nicht BH_4-sensitiven Phänotyp führen (Takarada *et al.* 1993; Takarada *et al.* 1994; Reblova *et al.* 2013).

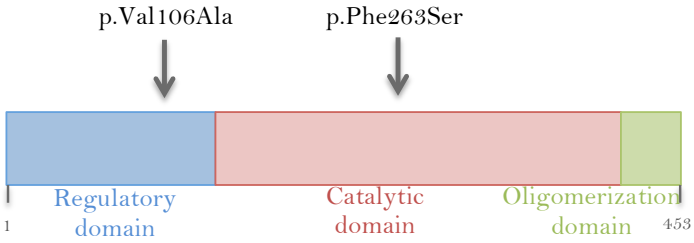

Abbildung 8. Mutationen in *Mm Pah*. p.Val106Ala (*Pah*$^{enu1/enu1}$) im Bereich der regulatorischen Domäne sowie p.Phe263Ser (*Pah*$^{enu2/enu2}$) im Bereich der katalytischen Domäne.

In der Leber findet sich keine messbare Enzymaktivität, und unter normaler Ernährung haben die *Pah*$^{enu2/enu2}$-Mäuse stark erhöhte Phenylalaninkonzentrationen im Plasma (Sarkissian *et al.* 2000). Basale Tätigkeiten wie adäquate Körperhygiene, Schwimm-Tests und verschiedene Konzentrations- und

Gedächtnisaufgaben sind beeinträchtigt (Shedlovsky *et al.* 1993; Zagreda *et al.* 1999). Des Weiteren bestehen ein verlangsamtes Wachstum, Mikrozephalie sowie, im Vergleich zu *Pah*$^{enu1/enu1}$ und *Pah*$^{enu1/enu2}$, eine Hypopigmentierung des Fells und der Augen (Abb. 9). Bei gestörtem Phenylalaninstoffwechsel in der Schwangerschaft kommt es zu einem maternalen PKU-Syndrom (Shedlovsky *et al.* 1993; McDonald 1994) mit Fehlbildungen und erhöhter Mortalität des Nachwuchses. Analysen des Hirngewebes (Martynyuk *et al.* 2010) zeigten Veränderungen im Neurotransmitter-Stoffwechsel, in der Nervenzell-Vernetzung und im Myelin-Aufbau (Puglisi-Allegra *et al.* 2000; Smith and Kang 2000; Yarotskyy *et al.* 2005; Winn *et al.* 2016) bei verändertem Proteinexpressions-Muster in den Zellen des Zentralen Nervensystems (Imperlini *et al.* 2014).

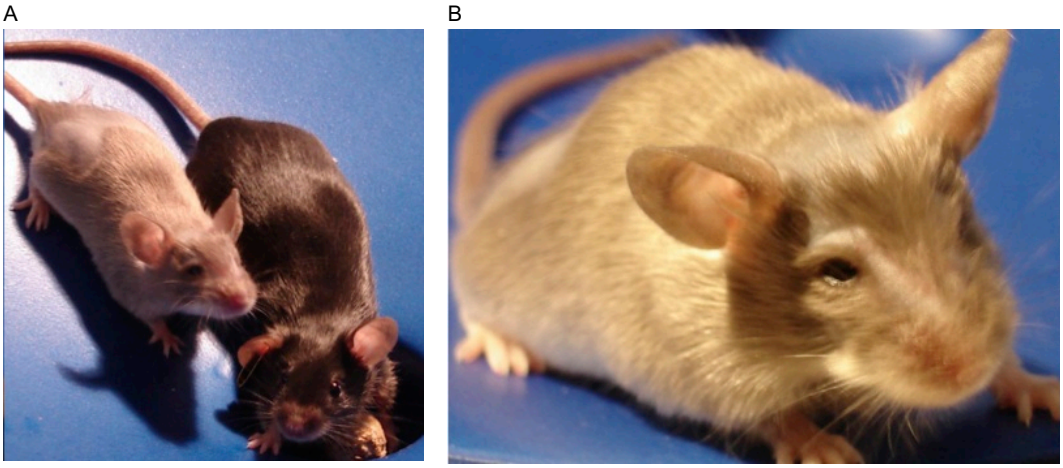

Abbildung 9. ENU-Mäuse: *Pah*$^{enu1/enu1}$ und *Pah*$^{enu2/enu2}$.
(A) *Pah*$^{enu2/enu2}$ (links, hell) im Vergleich zu *Pah*$^{enu1/enu1}$-Maus (rechts, dunkel).
(B) *Pah*$^{enu2/enu2}$-Maus mit dem typischen hellen und struppigen Fell.
(Abbildungen freundlicherweise von PD Dr. med. Lagler, Universität Innsbruck, zur Verfügung gestellt.)

Der Konduktor *Pah*$^{wt/enu2}$ hat einen unauffälligen Phänotyp bei einer Pah-Aktivität von ~ 50 % im Lebergewebe(Sarkissian *et al.* 2000). In neueren Studien, wobei hier die L-Phe-Werte der untersuchten *Pah*$^{wt/enu2}$-Mäuse im Serum im Bereich einer milden HPA lagen, zeigten sich allerdings Auffälligkeiten in spezifischen neurologischen Teilleistungsaspekten (Pascucci *et al.* 2013).

1.4.2.4 Das Mausmodell *Pah*$^{enu1/enu2}$ (ENU1/2)

Pah$^{enu1/enu2}$ (ENU1/2; [p.Val106Ala/p.Phe263Ser]) zeigt einen intermediären Phänotyp (Sarkissian *et al.* 2000). Wie der Großteil der Patienten ist dieser Genotyp *compound*-heterozygot, was die Variante zu einem besonders interessanten Krankheitsmodell macht.
Die L-Phe-Konzentrationen im Plasma sind mit Werten von 200 bis 300 µM nur leicht erhöht. Die Pah-Aktivität in der Leber liegt zwischen 5 und 25 % der Aktivität des Wildtyps (Sarkissian *et al.* 2000; Lagler *et al.* 2010).

1.4.2.5 BH_4-sensitive PKU-Mausmodelle

Bereits vor einigen Jahren konnte gezeigt werden, dass pharmakologische Dosen des Kofaktors auch die Menge an Wildtyp-Pah in der Mäuseleber erhöhen (Thöny *et al.* 2004; Scavelli *et al.* 2005).
Unsere Arbeitsgruppe konnte in Kooperation mit PD Dr. Florian Lagler (Universität Innsbruck) zeigen, dass *Pah*$^{enu1/enu1}$ und *Pah*$^{enu1/enu2}$ geeignete Modelle für die BH_4-sensitive PKU/HPA sind (Gersting *et al.* 2010; Lagler *et al.* 2010; Sarkissian *et al.* 2012). *Pah*$^{enu1/enu2}$ wurde bereits erfolgreich

eingesetzt, um die Wirkung weiterer pharmakologischer Chaperone zur Behandlung der PKU zu untersuchen (Santos-Sierra *et al.* 2012).

2 Ziele der Arbeit

Im Rahmen der klinischen Studie von Muntau *et al.* 2002 konnte gezeigt werden, dass eine bestimmte Gruppe von Patienten mit Phenylketonurie (PKU) von einer pharmakologischen Behandlung mit Tetrahydrobiopterin (BH_4), dem natürlichen Kofaktor des Enzyms Phenylalaninhydroxylase (PAH), profitiert. Somit konnte eine pharmakologische Alternative zur bisher etablierten belastenden diätetischen Therapie entwickelt werden (Muntau *et al.* 2002).
In der Arbeitsgruppe *Muntau/Gersting* wurden die Pathomechanismen des Enzymdefekts der PKU auf struktureller Ebene am gereinigten, rekombinant exprimierten PAH-Protein untersucht. Der Funktionsverlust der PAH bei Vorliegen von *Missense*-Mutationen im *PAH*-Gen ist häufig Folge einer Proteinfehlfaltung – also einer gestörten strukturellen Dynamik und einer konformationellen Instabilität des Proteins – und die Wirkungsweise des therapeutischen Effekts von BH_4 kann unter anderem auf eine Korrektur der Proteinfehlfaltung zurückgeführt werden. Ein gut charakterisiertes Tiermodell für die BH_4-Sensitivität stellt ein wichtiges Instrument dar, um die zellulären Mechanismen der Wirkung von BH_4 *in vivo* experimentell untersuchen zu können.
Das Ziel der vorliegenden Doktorarbeit war daher die Identifizierung und genauere Beschreibung BH_4-sensitiver Mausmodelle. Zunächst sollten die Wildtypen der murinen Pah und der humanen PAH hinsichtlich ihrer enzymkinetischen Parameter verglichen werden, um die Eignung der Maus als Modellorganismus für die PKU und den PAH-Defekt beim Menschen zu untersuchen. Im Weiteren sollte die bei den verfügbaren Mausmodellen vorliegenden varianten Pah-Enzyme (p.Va106Ala; p.Phe263Ser) genau charakterisiert werden und eine Korrelation mit dem klinischen Phänotyp der Mäuse untersucht werden. Neben den rekombinant gereinigten Proteinen sollte die Pah noch in weiteren zellfreien und zellulären Systemen untersucht werden – im zellfreien TnT-System, im eukaryoten Zellsystem mit Überexpression und in Leberlysaten der Mausmodelle. Als Marker für Proteinfehlfaltung sollten unter anderem eine Anfälligkeit gegenüber proteolytischem Verdau durch die Proteinase K, das Verhalten bei Hitzestress, sowie die Proteinkonformation mit Hilfe von Fluoreszenzmessungen und mittels Oligomerisierungsprofilen charakterisiert werden.
In einem weiteren Schritt sollten Veränderungen durch den Einfluss von pharmakologischen Dosen an BH_4 ermittelt werden. Bei Identifikation eines geeigneten Mausmodells für BH_4-Sensitivität sollte die Wirkung von BH_4 auf den Phenylalaninmetabolismus *in vivo* untersucht werden. Dabei sollte die Oxidationsrate von ^{13}C-markiertem Phenylalanin als Marker für die Aktivität der murinen Pah *in vivo* bestimmt werden und mit der Aktivität der Pah in isolierten murinen Hepatozyten verglichen werden.
Zusammenfassend war das Ziel der Doktorarbeit die eingehende Charakterisierung eines BH_4-sensitiven Mausmodells unter Verwendung von verschiedenen Modellsystemen *in vitro*, *ex vivo* und *in vivo*.

3 Arbeiten zur kumulativen Promotionsleistung

3.1 *Pah*$^{enu1/enu1}$ ist ein Mausmodell für die BH_4-sensitive Hyperphenylalaninämie und ermöglicht die *in-vivo*-Analyse des pharmakologischen Chaperon-Mechanismus

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* *contributed equally*

Für eine zunehmende Zahl an Erkrankungen konnte genetisch bedingte Proteinfehlfaltung – also eine Störung der dreidimensionalen Struktur von Proteinen aufgrund des Austauschs von Aminosäureseitenketten in der Proteinprimärstruktur – als molekularer Mechanismus der Krankheitsentstehung identifiziert werden. Zum Zeitpunkt der Veröffentlichung des vorliegenden Artikels waren mehr als 20 monogenetische Erkrankungen bekannt, für die Fehlfaltung und Funktionsverlust der durch genetische Variation betroffenen Proteine nachgewiesen wurde. In der Folge hat die Suche nach Möglichkeiten zur pharmakologischen Korrektur dieses Krankheitsmechanismus zunehmend an Bedeutung gewonnen. Untersuchungen *in vitro* hatten gezeigt, dass der Enzym-Kofaktor (6R)-L-*erythro*-5,6,7,8-Tetrahydrobiopterin (BH_4) zu einer Stabilisierung des fehlgefalteten Enzyms Phenylalaninhydroxylase (PAH) und somit zu einer Verminderung von Denaturierung und Degradation führt. Im Jahr 2008 wurde Sapropterin-Dihydrochlorid, die synthetische Form von BH_4, als pharmakologisches Chaperon zur Behandlung der BH_4-sensitiven Hyperphenylalaninämie (HPA) in Europa zugelassen (Kuvan®, Merck). Allerdings stand in der Zulassungsphase kein adäquates Tiermodell zur Verfügung, das den molekularen Mechanismus der BH_4-Sensitivität abbildete. Das Ziel der vorliegenden Veröffentlichung war daher, ein murines Modell zu identifizieren und den Krankheitsmechanismus der Proteinfehlfaltung sowie die molekularen Grundlagen der pharmakologischen Korrektur *in vitro* und *in vivo* zu untersuchen.
Es standen mit *Pah*$^{enu1/enu1}$ und *Pah*$^{enu2/enu2}$ durch Keimbahn-Mutagenese entstandene Modelle für genetische Variation des PAH-Enzyms zur Verfügung. Die homozygote Substitution p.Phe263Ser bei *Pah*$^{enu2/enu2}$ führt zu einem schweren Phänotyp des Tiermodells, der mit der klassischen Phenylketonurie bei Patienten vergleichbar ist, während die homozygote Substitution p.Val106Ala bei *Pah*$^{enu1/enu1}$ mit dem Phänotyp der milden HPA gleichzusetzen ist.
Wir konnten bei rekombinanter Expression von MBP-PAH-Fusionsproteinen ähnlich hohe spezifische Aktivitäten (V_{max}) für murine und humane Enzyme des jeweiligen Wildtyps (WT) zeigen. Die Analyse weiterer Parameter ergab, dass murine und humane PAH in Bezug auf die Bindung des Substrats Phenylalanin (L-Phe) ($[S]_{0,5}$; Substratkonzentration bei $V_{max}/2$), die Kooperativität der Bindung des Substrats (*h*, Hill-Koeffizient), die Bindung des Kofaktors (K_m; Dissoziationskonstante des Enzym-Kofaktor-Komplexes nach Michaelis-Menten) und die Aktivierung durch das Substrat eine vergleichbare Enzymkinetik aufwiesen. In den Lysaten der Lebergewebe lag allerdings bei der Maus eine doppelt so hohe Aktivität wie bei menschlichen Proben vor, wobei sich diese Unterschiede durch verschiedene hepatische PAH-Mengen erklären ließen. Alles in allem ist die Maus daher ein geeignetes Modell für das humane System der Phenylalanin-Hydroxylierung.
Die meisten enzymkinetischen Parameter der rekombinant exprimierten Mm MBP-Pah p.Val106Ala (spezifische Aktivität, positive Kooperativität, Affinität zu BH_4) sind mit dem murinen WT-Enzym

gut vergleichbar. Allerdings findet man bei p.Val106Ala eine leicht erhöhte Affinität zum Substrat L-Phe. Darüber hinaus befindet sich das variante murine Enzym in einem dauerhaft präaktivierten Zustand.

Bei Gabe von ^{13}C-markiertem Phenylalanin ermöglicht die kumulative $^{13}CO_2$-Ausscheidung in der Atemluft eine gute Abschätzung der Aktivität der Pah *in vivo*. Bei *Pah*$^{enu1/enu1}$-Tieren mit der Substitution p.Val106Ala lag die kumulative $^{13}CO_2$-Ausscheidung bei 33 % der WT-Rate; die Enzymfunktion war also *in vivo* deutlich erniedrigt im Vergleich zum rekombinant exprimierten und gereinigten Protein *in vitro*. Die *Pah*$^{enu1/enu1}$-Mäuse zeigten leicht erhöhte Phenylalaninkonzentrationen im Serum von ca. 190 µmol/l (Wildtyp, ~75 µmol/l). Die im Vergleich zum Wildtyp erniedrigte *in-vivo*-Aktivität spiegelte sich auch bei der Aktivitätsmessung im Leberlysat, sowie nach Expression im zellfreien TnT-System und in der Zellkultur (COS-7) wider. Des Weiteren war die absolute Menge des p.Val106Ala Pah-Proteins in der murinen Leber und bei Expression in eukaryoten COS-7-Zellen erniedrigt. Als Schlussfolgerung dieser Ergebnisse wurde ein Funktionsverlust des p.Val106Ala Pah-Enzyms bei *Pah*$^{enu1/enu1}$ als Folge einer verminderten Menge an p.Val106Ala Pah vermutet.

Im Oligomerisierungsprofil und in der nativen Gelelektrophorese (BN-PAGE) konnten wir im Vergleich zum WT eine erhöhte Menge an Aggregaten und an degradierten Fragmenten der p.Val106Ala Pah nachweisen. Eine erhöhte Hydrophobizität von p.Val106Ala verwies auf eine vermehrte Präsentation hydrophober Gruppen an der Oberfläche des Proteins. Diese Ergebnisse sprachen dafür, dass bei *Pah*$^{enu1/enu1}$ insgesamt eine erhöhte Menge an fehlgefalteter Pah vorliegt und diese anfälliger für Aggregation und Degradation ist als der Wildtyp.

Durch die Zugabe von BH_4 kam es zu einer Verminderung der Hydrophobizität an der Oberfläche der rekombinanten p.Val106Ala Pah; mit *Right Angle Light Scattering* (RALS) konnten wir eine Stabilisierung der Proteinaggregation mit einer deutlichen Abnahme der Aggregationskinetik nachweisen. Des Weiteren nahm die mittlere Entfaltungstemperatur bei Hitzestress um 2 °C zu.

Auch im Zellkulturmodell mit Expression der murinen Pah mit Substitution p.Val106Ala in COS-7-Zellen zeigte sich eine deutlich reduzierte Aggregation, wenn die Zellen mit einer Kombination von BH_4 und L-Phe inkubiert wurden. Bei der Therapie von *Pah*$^{enu1/enu1}$-Mäusen über drei Tage (BH_4, 10 µg/g Körpergewicht, zweimal pro Tag) kam es zu einem Anstieg der $^{13}CO_2$-Ausscheidungsraten im ^{13}C-Phenylalanin-Atemtest, also einer Zunahme der Enzymfunktion *in vivo*. In der Leber stieg die Menge an nachweisbarer Pah an und im Blut kam es zu einem signifikanten Abfall der Phenylalaninkonzentration. Insgesamt haben wir gezeigt, dass in Anwesenheit hoher BH_4-Konzentrationen die Konformation, also der Faltungszustand des Proteins, stabilisiert wird, was in Folge zu einer erhöhten Menge an funktionaler Pah führt. Dadurch kommt es bei *Pah*$^{enu1/enu1}$ zu einer Korrektur des biochemischen Phänotyps *in vivo*, wobei der therapeutische Effekt nur bei erhöhten Phenylalaninwerten im Serum eintrat und nicht beim gesunden Wildtyp zu einer Senkung der Phenylalaninkonzentration im Serum geführt hat.

Zusammenfassend sind murine Pah und humane PAH in Bezug auf ihre Enzymkinetik vergleichbar. Die murine Pah ist damit ein geeignetes Modell für die Analyse molekularer Mechanismen der Krankheitsentstehung und pharmakologischer Therapieoptionen bei HPA. Wir konnten homozygote *Pah*$^{enu1/enu1}$-Mäuse als geeignetes Modell für die BH_4-sensitive Phenylketonurie etablieren. Wie bei einer Vielzahl verschiedener humaner Varianten der PAH führte die Substitution p.Val106Ala nicht zu einem Verlust der spezifischen Aktivität, sondern zu einem Funktionsverlust aufgrund der verringerten Menge des Enzyms. Wir konnten als molekulare Ursachen Proteinfehlfaltung und Aggregation mit beschleunigter Degradation des Enzyms nachweisen. Die Anwesenheit hoher Konzentrationen von BH_4 führte zur Stabilisierung der Proteinkonformation und damit zu einer Erhöhung der Menge an funktionaler Pah. Die vorliegende Arbeit hat die molekularen Mechanismen des zugelassenen Wirkstoffs Sapropterin-Dihydrochlorid in Teilen aufgeklärt und so zu einem

besseren Verständnis der pharmakologischen Therapie von Proteinfehlfaltung durch kleine Moleküle beigetragen.

Eigener Beitrag zum Artikel:
Nach Einführung der benötigten Mutationen mit Hilfe der *Site Directed Mutagenesis* (für das humane Protein; murine Mutationen bereits vorliegend) wurden durch mich die Experimente mit prokaryoten Wildtyp-Proteinen und varianten Proteinen durchgeführt: Herstellung und Aufreinigung des prokaryoten Proteins (incl. Herstellung von SEC-Oligomerisationsprofilen), Durchführung nativer Gelelektrophoresen zur Beurteilung der Oligomerisierung, enzymkinetische Messungen, Proteolysen mit Hilfe von Proteinase K, Westernblots zur Quantifizierung von Proteinmengen, Fluoreszenzmessungen. Die Protein-Aufreinigungen wurden unter Mithilfe der naturwissenschaftlichen Doktorandinnen Marta K. Danecka und Dunja D. Reiß durchgeführt. Die Fluoreszenzmessungen erfolgten nach Protokollen und unter Aufsicht von Dunja Reiß. Des Weiteren führte ich die Expression der Proteine im eukaryoten System (COS-7), einschließlich des Proteinaufschluss, der Analyse der Proteinmengen sowie der Aktivitätsmessungen durch. Um eine weitere Bestätigung der Ergebnisse in einem eukaryoten System zu haben, erfolgte gemeinsam mit Dunja Reiß die Etablierung der TnT-Methode, in der erneut eine Analyse der Proteinmenge und Enzymaktivität erfolgte. Nach Erhalt der eingefrorenen Gesamtlebern (Tierhaltung im Kooperationslabor Lagler, Innsbruck) wurden diese nach einem von mir etablierten Protokoll aufgeschlossen und die Proteinmengen sowie Enzymaktivitäten vermessen. Bei der Manuskripterstellung wirkte ich im Bereich der Abbildungen, des Ergebnis- sowie des Methodenteils mit.

3.2 Die Tetrahydrobiopterin-Pharmakodynamik von *Pah*$^{enu1/enu2}$, dem *compound*-heterozygoten Modell für die BH_4-sensitive Hyperphenylalaninämie, ist Genotyp-spezifisch

Lagler, F. B.*, Gersting, S. W.*, Zsifkovits, C., Steinbacher, A., **Eichinger, A.**, Danecka, M. K., Staudigl, M., Fingerhut, R., Glossmann, H. and Muntau, A. C. (2010) New insights into tetrahydrobiopterin pharmacodynamics from *Pah*$^{enu1/2}$, a mouse model for compound heterozygous tetrahydrobiopterin-responsive phenylalanine hydroxylase deficiency. *Biochemical Pharmacology,* 2010, 80(10): 1563-1571.
* *contributed equally*

Die genetische Heterogenität ist bei Patienten mit Phenylketonurie besonders ausgeprägt. Neben der Vielzahl unterschiedlicher Mutationen trägt der Umstand, dass mehr als 80 % der Betroffenen *compound*-heterozygot sind, dazu bei. Dies ist auch bei PKU-Patienten mit BH_4-sensitivem Defekt der PAH der Fall.
Ziel dieser Arbeit war es, ein BH_4-sensitives Tiermodell zu etablieren, das – wie viele der Patienten – zwei unterschiedliche krankheitsauslösende Allele trägt. Hierzu haben wir die Mauslinien *Pah*$^{enu1/enu1}$ mit dem klinischen Phänotyp einer milden Hyperphenylalaninämie und die Mauslinie *Pah*$^{enu2/enu2}$ mit dem klinischen Phänotyp einer schwerwiegenden Phenylketonurie gekreuzt. Die daraus resultierende *compound*-heterozygote Mauslinie *Pah*$^{enu1/enu2}$ zeigte das Merkmal einer BH_4-Sensitivität. Dies eröffnete erstmals die Gelegenheit, die Pharmakodynamik und -kinetik einer BH_4-Therapie bei zwei unterschiedlichen BH_4-sensitiven Mausmodellen – der homozygoten *Pah*$^{enu1/enu1}$ und der *compound* -heterozygoten *Pah*$^{enu1/enu2}$ – zu vergleichen.
Für das Projekt wurde ein mehrstufiger methodischer Ansatz gewählt. Zunächst charakterisierten wir den biochemischen Phänotyp von *Pah*$^{enu1/enu1}$ und *Pah*$^{enu1/enu2}$, indem wir die Phenylalaninkonzentration und die Phenylalanin-Tyrosin-Ratio mit und ohne den Einfluss von BH_4 bestimmten.
In der Annahme, dass sich die beiden Tiermodelle in Bezug auf die Phenylalanin-*Clearance* unterscheiden, folgte die Untersuchung der Eliminationskinetik von Phenylalanin aus dem Blut. Darüber hinaus wendeten wir einen Pah-Enzymaktivitätstest *in vivo* an, bei dem den Tieren ^{13}C-Phenylalanin appliziert und die $^{13}CO_2$-Konzentration in der Ausatemluft bestimmt wurde. Diese ist ein Maß für die Phenylalaninoxidation *in vivo* und damit für den Schweregrad des enzymatischen Phänotyps. Da der Test in Abhängigkeit der Zeit durchgeführt wurde, erlaubte er die Bestimmung der Kinetik der Phenylalaninoxidation *in vivo* und damit einen Vergleich zwischen den beiden Tiermodellen. Sämtliche Experimente wurden im Vergleich mit einem Wildtyp-Mausstamm durchgeführt. Die Mausmodelle *Pah*$^{enu1/enu1}$ und *Pah*$^{enu1/enu2}$ unterschieden sich deutlich im Effektausmaß, der Pharmakodynamik sowie der Dosis-Wirkungs-Beziehung von BH_4. Bei *Pah*$^{enu1/enu2}$ zeigte sich im Vergleich zu P*ah*$^{enu1/enu1}$ ein geringerer Effekt pharmakologischer Dosen von BH_4 auf die Phenylalanin-Tyrosin-Ratio und auf die Phenylalanin-*Clearance*. Der Anstieg der Pah-Enzymaktivität im Atemtest war für *Pah*$^{enu1/enu2}$ zwar verzögert, im Ausmaß jedoch ähnlich zu *Pah*$^{enu1/enu1}$. Interessanterweise war die Dauer der Wirkung des Kofaktors beim *compound*-heterozygoten Genotyp deutlich kürzer als beim homozygoten milden Genotyp *Pah*$^{enu1/enu1}$. Die in den Mausmodellen *in vivo* gewonnenen Ergebnisse konnten am zellulären Modell bestätigt werden. Bei der Untersuchung der Pharmakokinetik lagen keine Unterschiede zwischen *Pah*$^{enu1/enu1}$ und *Pah*$^{enu1/enu2}$ vor. Diese Beobachtung erlaubt den Schluss, dass die Unterschiede in der Pharmakodynamik nicht auf Veränderungen der Aufnahme, Verteilung, Metabolisierung oder Ausscheidung des Wirkstoffs in den unterschiedlichen Tiermodellen beruhen.

Zusammenfassend ist es im Rahmen dieser Arbeit gelungen, ein *compound*-heterozygotes Mausmodell für den BH_4-sensitiven Defekt der PAH zu etablieren. Wir haben in Abhängigkeit des Genotyps klare Unterschiede in Bezug auf die Wirkung pharmakologischer Dosen des natürlichen Kofaktors des bei der PKU defekten Enzyms PAH festgestellt. Diese Ergebnisse können einen Beitrag zur Etablierung individualisierter Konzepte, sowohl für die Diagnostik als auch für die Genotyp-spezifische Therapie leisten. Dies ist in Anbetracht der Notwendigkeit einer weiteren Optimierung der medikamentösen Behandlung von PKU-Patienten von besonderer klinischer Bedeutung.

Eigener Beitrag zum Artikel:
Nach Einführung der Mutationen (p.Val106Ala, p.Phe263Ser, p.Phe263Leu) in die humane PAH mittels *Site Directed Mutagenesis* führte ich die PAH-Enzymaktivitätsmessungen an prokaryot exprimierter MBP-Pah durch. Darüber hinaus führte ich einen Teil der COS-7-Zellexperimente inklusive Kultivierung, PAH-Enzymaktivitätsmessungen und quantitativer PAH-Proteinbestimmung durch. Der restliche Anteil dieser Experimente wurde von der naturwissenschaftlichen Doktorandin Marta Danecka durchgeführt. An der Manuskripterstellung wirkte ich aktiv mit. Unter der Betreuung von Professor Gersting erstellte ich die Abbildungen zu den von mir durchgeführten Experimenten und die Kapitel zu den angewandten Methoden sowie einen Teil der Ergebniskapitel und der Diskussion.

3.3 Sekundärer BH_4-Mangel in Mäusen mit Fehlfaltung der PAH als mechanistische Verbindung der Proteinhomöostase mit der Regulation des Phenylalaninstoffwechsels

Eichinger, A.*, Danecka, M. K.*, Möglich, T., Borsch J., Woidy, M., Büttner, L., Muntau, A. C., Gersting, S. W. (2018) Secondary BH_4 deficiency links protein homeostasis to regulation of phenylalanine metabolism. *Human Molecular Genetics*, 2018, 27(10): 1732-1742.
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Die Verfügbarkeit der essentiellen Aminosäure Phenylalanin hängt im Wesentlichen von der Aufnahme durch die Nahrung und den Abbau durch das Phenylalanin-hydroxylierende System in der Leber ab. Geschwindigkeitsbestimmend ist die Enzymaktivität der PAH, die durch das Verhältnis von Phenylalanin zum Enzym-Kofaktor BH_4 reguliert wird. Phenylalanin bindet an eine allosterische Aktivierungsstelle am N-Terminus des Enzyms und führt so bei höheren Konzentrationen zur Aktivierung der PAH. Wenn der Kofaktor BH_4 im katalytischen Zentrum bei niedrigen Phenylalaninkonzentrationen an das allosterisch aktivierte Enzym bindet, findet durch eine konformationelle Umlagerung des N-Terminus eine Blockierung der allosterischen Phenylalanin-Bindungsstelle statt. In der Folge wird die PAH inhibiert und konformationell stabilisiert. In vorausgehenden Studien konnte gezeigt werden, dass *PAH*-Mutationen häufig zu fehlerhafter Faltung und Destabilisierung der PAH führen. Für einen Teil der Patienten mit genetischem Defekt der PAH konnte eine konformationelle Stabilisierung des Enzyms durch Bindung von BH_4 nachgewiesen werden.
Die Phenylalaninkonzentrationen in Blut und Gewebe unterliegen, bedingt durch Aufnahme und Abbau, – auch bei gesunden Personen – deutlichen Schwankungen, die bis zum 10-fachen über dem Normalwert reichen. Die Konzentration des Enzym-Kofaktors BH_4 wird in der Leberzelle jedoch konstant bei etwa 10 µM gehalten. Während der Behandlung von PKU-Patienten mit pharmakologischen Dosen von BH_4 kommt es zur Schwankung der BH_4-Konzentrationen in Blut und Gewebe. Die Verfügbarkeit von BH_4 in Leberzellen wurde jedoch noch nicht in Abhängigkeit des Genotyps bei varianter PAH untersucht.
In der vorliegenden Arbeit wurde zunächst die BH_4-Konzentration in Leberzellen von Mäusen mit mildem (*Pah*$^{enu1/enu1}$), schwerem (*Pah*$^{enu2/enu2}$) oder intermediärem (*Pah*$^{enu1/enu2}$) Phänotyp untersucht. Bei Anwesenheit des *enu1*-Allels zeigen die Mäuse einen sekundären hepatischen BH_4-Mangel, also eine verminderte Konzentration des freien Enzym-Kofaktors in Hepatozyten. Eine verminderte Bildung des Kofaktors BH_4 konnte mittels quantitativer mRNA-Analyse von *Gch1*, dem wichtigsten Enzym der BH_4-Synthese, ausgeschlossen werden. Ebenso konnte kein erhöhter Verbrauch des Kofaktors durch eine entkoppelte enzymatische Reaktion gezeigt werden.
Um die unterschiedlichen BH_4-Konzentrationen in Leberzellen der murinen Modelle in der Zellkultur untersuchen zu können, wurden die varianten Enzyme in COS-7-Zellen in Anwesenheit von Sepiapterin, einer Vorstufe von BH_4, exprimiert. Auch hier zeigte sich eine reduzierte Verfügbarkeit von freiem, intrazellulärem BH_4 für die p.Val106Ala Pah exprimierenden Zellen. Dies zeigte sich besonders ausgeprägt nach einer im Anschluss durchgeführten Sepiapterin-freien Inkubation der Zellen. Durch Analyse prokaryot exprimierter, aufgereinigter Pah wurde gezeigt, dass durch Mutationen konformationell destabilisierte und fehlgefaltete Enzyme den Kofaktor verstärkt zu binden scheinen und damit die Konzentration an freiem BH_4 reduzieren. Die Verschiebung des Verhältnisses von freiem zu gebundenem BH_4 führt zu einer Störung der durch das Verhältnis von Phenylalanin zu BH_4 gesteuerten Regulation der Pah.

Damit wurde in dieser Arbeit ein Mechanismus gezeigt, wie durch genetisch bedingte Änderung der Proteinfaltung und damit des hepatischen Mikromilieus eine Störung der Regulation von Metaboliten-Stoffwechselwegen entstehen kann. Die Betrachtung komplexer Gen-Umwelt- und Gen-Medikament-Interaktion kann einen wichtigen Beitrag dazu leisten, die zellulären Grundlagen von Stoffwechselregulation und -adaptation sowohl unter physiologischen als auch unter pathologischen Bedingungen zu verstehen.

Eigener Beitrag zum Artikel:
Die Tierhaltung sowie Aufreinigung der primären Hepatozyten wurde von Tamara M. Möglich sowie Julia Borsch durchgeführt. Durch mich wurden die murinen Proben weiterverarbeitet und die biochemischen und biophysikalischen Experimente *ex vivo* Versuche durchgeführt. Hierzu habe ich die BH_4/BH_2-Messung, die auch in den weiteren Versuchen zur Anwendung kam, sowie die *Real-Time*-PCR etabliert. Gemeinsam mit Marta K. Danecka, die die *in vitro* Versuche durchführte, erfolgte die Erarbeitung einer ersten Version des Manuskripts des veröffentlichten *Papers*, welches unter der Betreuung von Professor Muntau und Professor Gersting weiterbearbeitet wurde.

3.4 Poster

Eichinger A, Danecka MK, Reiß DD, Woidy M, Staudigl M, Gersting SW, Muntau AC: BH_4-responsive mouse models in phenylketonuria – mutual impact of genotype, phenylalanine substrate and BH_4 cofactor on enzyme function. Barcelona, Spain, Poster Presentation SSIEM 2013.

4 Zusammenfassung in deutscher und englischer Sprache

4.1 Zusammenfassung

Phenylketonurie (PKU) ist eine angeborene Störung des Aminosäurestoffwechsels, die unbehandelt zu schweren neurologischen Schäden führt. In vielen Fällen ist die einzige mögliche Behandlungsoption eine streng eiweißarme Diät. Im Rahmen klinischer Studien konnte gezeigt werden, dass etwa ein Drittel der Patienten auf die Gabe pharmakologischer Dosen des natürlichen Kofaktors der Phenylalaninhydroxylase (PAH), Tetrahydrobiopterin (BH_4), ansprechen (Muntau *et al.* 2002). Mit der Hilfe von BH_4 ergab sich eine Behandlungsalternative, die den Patienten erlaubte ihre Diät zu lockern und mehr natürliches Protein mit der Nahrung aufzunehmen (Muntau *et al.* 2002). Zentrale Fragestellung der vorliegenden Doktorarbeit war die Identifikation und eingehende Charakterisierung BH_4-sensitiver Mausmodelle. Das homozygote Mausmodell *Pah*$^{enu1/enu1}$ trägt die Variante p.Val106Ala der murinen Phenylalaninhydroxylase (Pah). Der Aminosäureaustausch beeinträchtigt die regulatorische Domäne und führt zu einem milden PKU-Phänotyp. Das homozygote Mausmodell *Pah*$^{enu2/enu2}$ ist Träger der Mutation p.Phe263Ser, die in der katalytischen Domäne des Enzyms gelegen ist und zum klinischen Bild einer schweren PKU führt. *Pah*$^{enu1/enu2}$ zeigt als *compound*-heterozygotes Mausmodell einen intermediären klinischen Phänotyp. Die Aminosäuren Phe 263 und Val 106 sind zwischen der murinen Pah und der humanen PAH konserviert. Wie im entsprechenden Mausmodell führt die Variante p.Val106Ala beim Menschen zu einem milden Phänotyp und p.Phe263Ser zu einem schweren Phänotyp. Bei Analyse der enzymkinetischen Parameter am rekombinanten Protein (Wildtyp, p.Val106Ala, p.Phe263Ser) konnte eine gute Vergleichbarkeit zwischen der murinen und humanen PAH gezeigt werden. Unterschiede in den Enzymaktivitäten zwischen Proben von murinen und humanen Leberlysaten beruhen auf unterschiedlichen hepatischen PAH-Mengen. Die im Rahmen der Arbeit erhobenen Daten zeigten, dass die murine Pah ein gut geeignetes Modell für das PAH-System des Menschen ist.
Sowohl für *Pah*$^{enu1/enu1}$ als auch für *Pah*$^{enu1/enu2}$ konnten wir nachweisen, dass diese Mausmodelle von einer pharmakologischen Therapie mit BH_4 profitieren und somit eine BH_4-Sensitivität vorliegt. *Pah*$^{enu2/enu2}$ zeigte kein Ansprechen auf die BH_4-Therapie. Am rekombinant hergestellten Protein führte die Variante p.Val106Ala *in vitro* weder zu einer Verminderung der Affinität zum Kofaktor noch zu einer Reduktion der spezifischen Enzymaktivität. Somit kann die Wirkung von BH_4 nicht durch eine direkte Wirkung als Kofaktor der enzymatischen Reaktion erklärt werden. In biophysikalischen und biochemischen Experimenten an gereinigten Proteinen und im zellulären Modell konnte gezeigt werden, dass die Variante p.Val106Ala zu vermehrter Aggregation und Degradation neigt und damit zu einer Verminderung des intakten Enzyms führt. Auf molekularer Ebene induzierte BH_4 eine Korrektur der p.Val106Ala-Proteinfehlfaltung und führte somit, als sogenanntes pharmakologisches Chaperon, zu einer Erhöhung der effektiven intrazellulären Konzentration von korrekt gefalteter und funktionaler Pah. Beim Vergleich der pharmakokinetischen und -dynamischen Parameter einer BH_4-Therapie von *Pah*$^{enu1/enu1}$ und *Pah*$^{enu1/enu2}$ haben wir Unterschiede zwischen diesen beiden BH_4-sensitiven Mausmodellen festgestellt. Dies zeigt, dass die BH_4-Wirkung nicht nur von der BH_4-sensitiven Variante (hier p.Val106Ala) bestimmt wird, sondern auch vom zweiten Allel beeinflusst wird. Dieser Effekt der interallelischen Komplementation sollte bei den *compound*-heterozygoten Patienten berücksichtigt werden und könnte erweiterte Testprotokolle notwendig machen.
Im Rahmen der *ex vivo* Experimente wurden bei nicht diätetisch oder pharmakologisch therapierten Mäusen Unterschiede in den freien, intrazellulären BH_4-Konzentrationen zwischen den verschiedenen Mausgenotypen beobachtet. Interessanterweise waren diese Unterschiede der BH_4-Konzentration nicht im Blut zu beobachten. Die intrazelluläre BH_4-Konzentration von Hepatozyten

war bei *Pah*$^{enu1/enu1}$ und *Pah*$^{enu1/enu2}$ im Vergleich zum Wildtyp sowie zu *Pah*$^{enu2/enu2}$ erniedrigt. Es konnten keine Unterschiede in der Expression des Schlüsselenzyms der Tetrahydrobiopterin-Biosynthese, *Gch1*, nachgewiesen werden. Darüber hinaus konnte ein ungehemmter BH_4-Verbrauch im Rahmen eines enzymatischen *Uncoupling* für die *Pah*^{enu1}-Allel tragenden Mäuse ausgeschlossen werden. Wir konnten *in vitro* zeigen, dass zwar kein Unterschied in der absoluten BH_4-Konzentration vorliegt, jedoch eine Verschiebung innerhalb des BH_4-Pools stattfindet, was zu einer Reduktion des freien BH_4 führte. Bei *Pah*$^{enu1/enu1}$ und *Pah*$^{enu1/enu2}$, die das Allel p.V106Ala tragen, scheint es in den Hepatozyten zu einer verstärkten Bindung von BH_4 an die Pah (*trapping*) zu kommen, so dass BH_4 nicht mehr frei verfügbar in der Zelle vorliegt. Somit kommt es neben dem mutationsbedingten Funktionsverlust der p.Val106Ala-Pah auch zu einem sekundären BH_4-Mangel, der zu einer weiteren Verminderung der Enzymaktivität beitragen könnte. Die Gabe von pharmakologischen Dosen von BH_4 könnte neben der Korrektur der Proteinfehlfaltung als pharmakologisches Chaperon somit auch einen Ausgleich des sekundären intrazellulären BH_4-Mangels bewirken.
Ein besseres Verständnis der molekularen Mechanismen der Krankheitsentstehung und der pharmakologischen Korrektur bei den BH_4-sensitiven Mausmodellen *Pah*$^{enu1/enu1}$ und *Pah*$^{enu1/enu2}$ kann dazu beitragen, die pharmakologische Therapie, zum Beispiel durch die Entwicklung von optimierten oder neuen Wirkstoffen, zu verbessern und effizienter zu machen. Darüber hinaus können diese Mausmodelle auch genutzt werden, um grundsätzliche Fragestellungen zu Proteinfehlfaltungserkrankungen und Degradationsmechanismen zu adressieren. So könnten die Mausmodelle bei Untersuchungen der zellulären Degradationsmaschinerie, des Einflusses von oxidativem Stress auf Proteinfaltung und der Korrektur der gestörten Proteinhomöostase mittels Proteostase-Regulatoren verwendet werden.

4.2 Summary

Phenylketonuria (PKU) is an inborn error of amino acid metabolism which causes severe neurological impairment, if untreated. For most patients, a strict low-phenylalanine diet is the only treatment option. In clinical studies, it was shown that approximately one third of the patients respond to supplementation with pharmacological dosages of tetrahydrobiopterin (BH_4), which is the natural cofactor of the enzyme phenylalanine hydroxylase (PAH). Treatment with BH_4 gave rise to a pharmacological therapeutic alternative that allows patients to relax their diet and tolerate more natural protein (Muntau *et al.* 2002). The main topic of this thesis was the identification and the detailed characterization of a BH_4-responsive mouse model. The homozygous *Pah*$^{enu1/enu1}$ mouse carries the allelic variant p.Val106Ala of murine Pah. The amino acid side chain replacement affects the regulatory domain leading to a mild PKU phenotype. The homozygous *Pah*$^{enu2/enu2}$ mouse carries the allelic variant p.Phe263Ser, which maps to the catalytic domain of the enzyme and causes a clinical picture of severe PKU. The compound heterozygous *Pah*$^{enu1/enu2}$ mouse shows an intermediate clinical phenotype. The amino acids Phe 263 and Val 106 of the PAH protein are conserved between humans and mice. According to phenotypes observed in mice, p.Val106Ala leads to a mild phenotype in humans and p.Phe263Ser induces classical PKU. Enzyme kinetics analyses using recombinantly expressed protein (wild-type, p.Val106Ala, p.Phe263Ser) showed good comparability between human and murine PAH. The differences between the activity of human and murine PAH in the liver tissues seemed to be due to different intrahepatic PAH amounts. The data presented here shows that murine Pah is an appropriate model for the human phenylalanine hydroxylating system.
Both *Pah*$^{enu1/enu1}$ and *Pah*$^{enu1/enu2}$ showed BH_4 responsiveness and benefit from treatment with BH_4, whereas *Pah*$^{enu2/enu2}$ showed no response to BH_4 treatment. *In vitro*, the recombinantly expressed p.Val106Ala protein showed neither a reduction of affinity to its cofactor BH_4 nor a marked reduction in specific enzyme activity. Thus, BH_4 responsiveness cannot be explained by a direct

cofactor effect on the enzymatic reaction. Detailed biophysical and biochemical investigation using purified protein and eukaryotically expressed protein showed propensity towards degradation and aggregation for p.Val106Ala, leading to a decrease in the available amount of functional protein. On a molecular level, BH_4 rescued protein misfolding of p.Val106Ala, acting as a pharmacological chaperone. BH_4 enhanced the amount of correctly folded and enzymatically active Pah. Comparison of pharmacokinetics and pharmacodynamics of BH_4 therapy in *Pah*$^{enu1/enu1}$ and *Pah*$^{enu1/enu2}$ showed differences between genotypes. The BH_4 effect is not only determined by one allele, *e.g.* a BH_4-responsive variant (here p.Val106Ala), but also by the second allele (p.Val106Ala or null mutation p.Phe263Ser). These differences in response to the BH_4 therapy may lead to the need for expanded testing in compound heterozygous patients.

In *ex vivo* analyses, we observed differences in hepatic free intracellular BH_4 between untreated genotypes. Interestingly, this was not congruent with the BH_4 pattern in blood. The BH_4 concentration in hepatocytes was decreased for *Pah*$^{enu1/enu1}$ and *Pah*$^{enu1/enu2}$ in comparison to the wild-type and *Pah*$^{enu2/enu2}$. No differences were found in the expression of *Gch1*, the key enzyme of tetrahydrobiopterin biosynthesis. Moreover, no increase in the consumption of BH_4 due to enzymatic uncoupling was observed for genotypes carrying the *Pah*^{enu1} allele. We demonstrated *in vitro* that the total amount of BH_4 was not altered, but a shift in BH_4 pools led to a decrease in free BH_4. Misfolded p.Val106Ala Pah protein trapped BH_4 making it unavailable for the enzymatic reaction. In addition to the loss of function due to the mutation p.Val106Ala, this mechanism may lead to secondary intracellular BH_4 deficiency, which induces a further decrease in enzyme function. Therefore, therapeutic BH_4 may not only exert a pharmacological chaperone effect on the misfolded p.Val106Ala protein, the treatment may also correct intrahepatic secondary BH_4 deficiency.

A better understanding of the molecular mechanisms of BH_4-responsive PKU could lead to optimization of the current therapy with BH_4 and might enable the identification of new and more efficient pharmacological compounds. Moreover, mice carrying the *Pah*^{enu1} allele could be used to address more general questions regarding diseases with protein misfolding and degradation. For example, the mouse model could help to elucidate mechanisms of intracellular protein degradation, the impact of oxidative stress on protein misfolding and therapeutic routes to correct protein homeostasis by proteostasis regulation.

5 Literaturverzeichnis und Datenbanken

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5.2 Datenbanken

BIOPKUdb	Database of PKU genotypes investigated for BH_4-responsiveness www.biopku.org, www.bh4.org/biopku.html
HGMD	The Human Gene Mutation Database At the Institute of Medical Genetics in Cardiff www.hgmd.org
OMIM	Online Mendelian Inheritance in Man OMIM Entry # 261600 www.omim.org
ExPASy	Expert Protein Analysis System EC 1.14.16.1 (phenylalanine 4-monooxygenase) www.expasy.org

6 PAH-Sequenzen, Abkürzungen und Nomenklatur

6.1 PAH-Sequenzen

Tabelle 1) Sequenzen der **humanen PAH** (hs PAH) und der **murinen Pah** (Mm Pah).

	PAH [Homo sapiens]	PAH [Mus musculus]
Gen	Gene ID: 5053 http://www.ncbi.nlm.nih.gov/gene/5053	Gene ID: 18478 http://www.ncbi.nlm.nih.gov/gene/18478
Lage	12q22-q24.2; NC_000012.11 (133 851 895 bp) http://www.ncbi.nlm.nih.gov/nuccore/NC_000012.1	Chr. 10 C2-D1; 10 43.64 cM; NC_000076.6 http://www.ncbi.nlm.nih.gov/nuccore/NC_000076.6
gDNA	NG_008690.1 (86 277 bp DNA) http://www.ncbi.nlm.nih.gov/nuccore/NG_008690.1	NC_000076.6 (62 343 bp DNA)
mRNA	NM_000277.1 (2 680 bp mRNA) http://www.ncbi.nlm.nih.gov/nuccore/NM_000277.1	NM_008777.3 (2 152 bp mRNA) http://www.ncbi.nlm.nih.gov/nuccore/NM:008777.3
Protein	NP_000268.1 (452 AS) http://www.ncbi.nlm.nih.gov/protein/NP_000268.1	NP_032803.2 (453 AS) http://www.ncbi.nlm.nih.gov/protein/NP_032803.2

6.2 Aminosäure-Nomenklatur und Abkürzungen

Tabelle 2) Nomenklatur Aminosäuren. Drei- und Einbuchstabencode.

Aminosäure	Dreibuchstabencode	Einbuchstabencode
Arginin	Arg	R
Asparagin	Asn	N
Aspartat	Asp	D
Cystein	Cys	C
Glutamin	Gln	Q
Glutamat	Glu	E
Glycin	Gly	G
Histidin	His	H
Isoleucin	Ile	I
Leucin	Leu	L
Lysin	Lys	K
Methionin	Met	M
Phenylalanin	Phe	F
Prolin	Pro	P
Serin	Ser	S
Threonin	Thr	T
Tryptophan	Trp	W
Tyrosin	Tyr	Y
Valin	Val	V

Tabelle 3) Abkürzungen.

AAH	Aromatische Aminosäure Hydroxylase
Abb.	Abbildung
AS	Aminosäure
BH_2	Dihydrobiopterin
BH_4	(6R)-L-*erythro*-5,6,7,8-Tetrahydrobiopterin
Bp	Basenpaar(e)
Chr.	Chromosom
Da	Dalton
gDNA	Genomische DNA
GFRP	GTP Cyclohydrolase Feedback Regulatory Protein
GTP	Guanosintriphosphat
GTPCH	GTP-Cyclohydrolase
HPA	Hyperphenylalaninämie
hs	human/menschlich
K_M	Affinität zum Kofaktor BH_4
LNAA	*Large Neutral Amino Acids*
MBP	*Maltose Binding Protein*
Mm	murin/mäuslich
mRNA	*Messenger* RNA
O_2	Sauerstoff
Pah	Murine Phenylalaninhydroxylase (Enzym)
Pah	*Murines Phenylalaninhydroxylase*-Gen
PAH	Humane Phenylalaninhydroxylase (Enzym)
PAH	*Humanes Phenylalaninhydroxylase*-Gen
PKU	Phenylketonurie
PTPS	6-Pyruvoyl-5,6,7,8-Tetrahydrobiopterin-Synthase
SEC	Size Exclusion Chromatography
SR	Sepiapterin Reduktase
Tbl.	Tabelle
WT	Wildtyp

7 Sonderdrucke der Publikationen im Rahmen der Promotionsarbeit

Gersting, S. W.*, Lagler, F. B.*, **Eichinger, A.**, Kemter, K. F., Danecka, M. K., Messing, D. D., Staudigl, M., Domdey, K. A., Zsifkovits, C., Fingerhut, R., Glossmann, H., Roscher, A. A. and Muntau, A. C. (2010) *Pah^{enu1}* is a mouse model for tetrahydrobiopterin-responsive phenylalanine hydroxylase deficiency and promotes analysis of the pharmacological chaperone mechanism in vivo. *Human Molecular Genetics*, 2010, 19(10): 2039-2049.
* *contributed equally*

Lagler, F. B.*, Gersting, S. W.*, Zsifkovits, C., Steinbacher, A., **Eichinger, A.**, Danecka, M. K., Staudigl, M., Fingerhut, R., Glossmann, H. and Muntau, A. C. (2010) New insights into tetrahydrobiopterin pharmacodynamics from *Pah$^{enu1/2}$*, a mouse model for compound heterozygous tetrahydrobiopterin-responsive phenylalanine hydroxylase deficiency. *Biochemical Pharmacology*, 2010, 80(10): 1563-1571.
* *contributed equally*

Eichinger, A.*, Danecka, M. K.*, Möglich, T., Borsch J., Woidy, M., Büttner, L., Muntau, A. C., Gersting, S. W. (2018) Secondary BH_4 deficiency links protein homeostasis to regulation of phenylalanine metabolism. *Human Molecular Genetics*, 2018, 27(10): 1732-1742.
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Pah^{enu1} is a mouse model for tetrahydrobiopterin-responsive phenylalanine hydroxylase deficiency and promotes analysis of the pharmacological chaperone mechanism *in vivo*

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The recent approval of sapropterin dihydrochloride, the synthetic form of 6[R]-L-*erythro*-5,6,7,8-tetrahydrobiopterin (BH_4), for the treatment of phenylketonuria (PKU) as the first pharmacological chaperone drug initiated a paradigm change in the treatment of monogenetic diseases. Symptomatic treatment is now replaced by a causal pharmacological therapy correcting misfolding of the defective phenylalanine hydroxylase (PAH) in numerous patients. Here, we disclose BH_4 responsiveness in *Pah*^{enu1}, a mouse model for PAH deficiency. Loss of function resulted from loss of PAH, a consequence of misfolding, aggregation, and accelerated degradation of the enzyme. BH_4 attenuated this triad by conformational stabilization augmenting the effective PAH concentration. This led to the rescue of the biochemical phenotype and enzyme function *in vivo*. Combined *in vitro* and *in vivo* analyses revealed a selective pharmaceutical action of BH_4 confined to the pathological metabolic state. Our data provide new molecular-level insights into the mechanisms underlying protein misfolding with loss of function and support a general model of pharmacological chaperone-induced stabilization of protein conformation to correct this intracellular phenotype. *Pah*^{enu1} will be essential for pharmaceutical drug optimization and to design individually tailored therapies.

INTRODUCTION

The biological and medical significance of protein misfolding as a basis of disease is increasingly recognized. Distinct pathophysiological mechanisms underlying protein misfolding disorders are known. In neurodegenerative diseases such as Alzheimer's or Parkinson's disease, misfolded proteins accumulate and form insoluble aggregates associated with molecular and clinical dysfunction (1–3). In other disorders, missense mutations cause unfavorable structural rearrangements resulting in conformational destabilization, rapid degradation and a lack of the functional protein (4–8). The latter group of protein misfolding diseases with loss of function now comes into focus of research. More than 20 monogenetic [e.g. cystic fibrosis, phenylketonuria (PKU)] and acquired (e.g. p53 mutations) disorders are known to be associated with this molecular phenotype (Supplementary Material, Table S1).

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Pharmacological chaperones are small-molecule chemical compounds that manipulate dysfunctional biological systems by stabilizing protein conformation in misfolding diseases (7–11). The first pharmacological chaperone drug, BH_4 (Kuvan®), was approved in 2008 for the treatment of PKU (MIM 261600), the most frequent genetic disorder of amino acid catabolism. Pharmacological doses of BH_4, the natural cofactor of the deficient enzyme phenylalanine hydroxylase (PAH), restore enzyme function by a mechanism other than its cofactor action in a significant share of patients (12). First *in vitro* studies using purified PAH pointed to stabilization of the misfolded protein against denaturation and degradation (13–16). In wild-type (17,18) and transgenic mice with deficiency in BH_4 biosynthesis (19), supplementation of BH_4 enhanced the amount of hepatic wild-type PAH protein and *in vivo* enzyme activity. However, an animal model showing the particular phenotype of BH_4-responsive PAH deficiency was not available for approval. This precluded the elucidation of the molecular mechanisms causing loss of function and the mode of action underlying pharmacological chaperone therapy *in vivo*. The aim of this study, therefore, was to identify a mouse model for BH_4-responsive PAH deficiency that would enable *in vitro* and *in vivo* experimental insight into the biological processes associated with PAH deficiency and into the mechanisms of therapeutic correction of the loss-of-function phenotype at the molecular level.

Two mouse strains produced by germline mutagenesis harboring defective *Pah* genes are available (20). The V106A mutation in Pah^{enu1} leads to a mild hyperphenylalaninemia phenotype (21), whereas Pah^{enu2} (F263S) shows severe PKU (www.pahdb.mcgill.ca) (20,22,23). Although Pah^{enu1} was thoroughly studied (20–24), it was not recognized to be a model for the new clinical entity of BH_4-responsive PKU.

The present study disclosed misfolding-induced PAH deficiency and its pharmacological rescue by BH_4 in Pah^{enu1}, reflecting the situation in human hyperphenylalaninemia. Besides accelerated degradation, we observed aggregate formation of the PAH protein *in vitro* and *in vivo*, revealing a novel pathophysiological aspect of protein misfolding in the particular group of conformational disorders with loss of function. Moreover, we showed that BH_4 corrects the biochemical phenotype by stabilizing the target protein conformation, and we retraced the mode of action of the pharmacological chaperone on misfolding, aggregation and degradation of the PAH protein *in vivo*. The animal model presented provided insights into general mechanisms underlying protein misfolding with loss of function and helped to understand the pharmacological chaperone effect of BH_4 at a molecular level. This knowledge will allow us to exploit the resources of chemical compound libraries to design future therapeutic agents for other protein misfolding diseases.

RESULTS

Murine PAH is a suitable model for the human phenylalanine hydroxylating system

It was previously hypothesized that the total activity of murine PAH is higher than that of human PAH due to differences in PAH regulation by substrate and cofactor (22,25). We re-evaluated whether *Mus musculus* is a suitable model for issues related to human PAH by generating the first complete kinetic data from recombinant murine PAH proteins.

Table 1. Steady-state enzyme kinetic parameters of human and murine PAH proteins

Species	Specific activity[a] (nmol Tyr/min × mg protein)	$[S]_{0.5}$[b] (μM)	K_m[c] (μM)	h[b]	Activation fold
WT-HsPAH	3115 ± 149	155 ± 6	24.0 ± 3	3.0	3.0
WT-MmPAH	2628 ± 293	175 ± 34	22.0 ± 5	2.6	3.1

Recombinant tetrameric human (WT-HsPAH) and murine (WT-MmPAH) wild-type PAH were expressed as MBP–PAH fusion proteins in *E. coli*. Apparent affinities for L-phenylalanine ($[S]_{0.5}$), BH_4 (K_m) and the Hill coefficient (h) as a measure of cooperativity are shown. Activation fold represents substrate activation as the ratio of specific activity with and without prior incubation with L-phenylalanine. Values are given as means ± SEM of $n = 3$ experiments.

[a]Specific activity was determined at standard L-phenylalanine (1 mM) and BH_4 (75 μM) concentrations with L-phenylalanine pre-incubation.

[b]Apparent affinities for L-phenylalanine ($[S]_{0.5}$) and the Hill coefficient (h) were determined at variable L-phenylalanine concentrations (0–4.5 mM) and standard BH_4 concentration (75 μM).

[c]Apparent affinities for BH_4 (K_m) were determined at variable BH_4 concentrations (0–700 μM) and standard L-phenylalanine concentration (1 mM).

The specific activity of tetrameric murine wild-type PAH expressed in *E. coli* as fusion protein with maltose binding protein (MBP) was well comparable to that of human wild-type PAH. Apparent affinities of the enzymes for the substrate L-phenylalanine and the BH_4 cofactor, cooperativity for substrate binding and substrate-induced enzyme activation did not differ significantly between murine and human PAH (Table 1). However, total PAH enzyme activity and the PAH protein amount in crude liver lysates of wild-type mice were about twice as high as in human liver (Fig. 1A). Thus, murine and human PAH show similar specific activity and analogous regulation, whereas the inconsistency between specific and total PAH enzyme activity is due to differences in the PAH protein amount *in vivo*.

Loss of function in Pah^{enu1} is associated with reduced amounts of PAH protein

Next, we related specific enzyme activity *in vitro* to residual protein amount and enzyme function *in vivo*. ^{13}C-phenylalanine breath tests assessing phenylalanine oxidation are a good measure for overall *in vivo* PAH activity in humans as well as in mice (12,17,26). The cumulative recovery of $^{13}CO_2$ representing ^{13}C-phenylalanine turnover was 33% for Pah^{enu1} and 12% for Pah^{enu2} when compared with wild-type mice. The mild reduction in enzyme activity in Pah^{enu1} still induced an increase in mean blood phenylalanine concentration to 189 μmol/l (wild-type 76 μmol/l), reflecting a mild hyperphenylalaninemia phenotype, whereas Pah^{enu2} showed phenylalanine concentrations in the range of classical PKU (1144 μmol/l) (Fig. 1B). Data for PAH activity and blood phenylalanine levels are in agreement with previous studies using crude liver lysates (17,22).

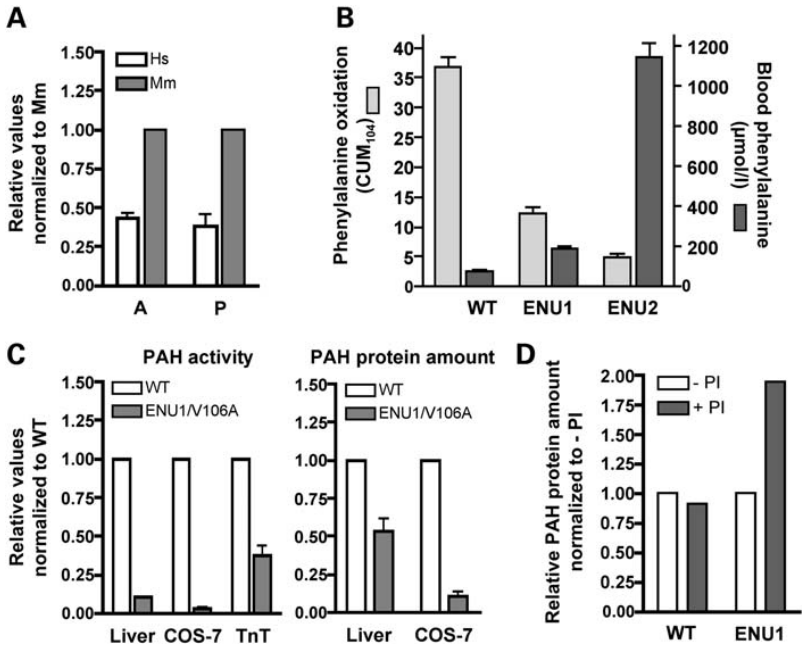

Figure 1. Comparison of human and murine PAH and characterization of PAH deficiency in *Pah*^{enu1} and *Pah*^{enu2} mice. (**A**) Relative PAH activity (A) and PAH protein amount (P) in human and murine crude liver lysates. Data were normalized to murine samples and are given as means ± s.e.m. of $n = 3$ experiments. (**B**) Phenylalanine oxidation and blood phenylalanine levels of wild-type (WT, $n = 6$), *Pah*^{enu1} (ENU1, $n = 10$) and *Pah*^{enu2} (ENU2, $n = 4$) mice *in vivo*. The phenylalanine oxidation rate was assessed by determination of the cumulative $^{13}CO_2$ recovery at 104 min (CUM_{104}) after i.p. application of ^{13}C-phenylalanine (15 µg/g body weight). Values are given as means ± s.e.m. (**C**) Relative PAH activity (left) and PAH protein amount (right) of murine wild-type PAH (WT) and ENU1/V106A-PAH in crude liver lysates, expressed in COS-7 cells and in the TnT system. Data were normalized to WT-PAH and are given as means ± s.e.m of $n = 3$ experiments. (**D**) Protection of PAH protein by protease inhibitors (PI). Liver lysates of wild-type mice (WT) and *Pah*^{enu1} (ENU1) mice in the absence or presence of PI. Data of $n = 1$ experiment were normalized to conditions without PI.

Recombinant expression and purification of variant murine PAH allowed for detailed analysis of the catalytic function of *Pah*^{enu1} (V106A) and *Pah*^{enu2} (F263S). The latter harbors a mutation in the catalytic core, leading to a near-complete loss of specific PAH activity, whereas V106A undergoes loss of function by distinct molecular mechanisms. Compellingly, the V106A replacement in the regulatory domain resulted in unimpaired specific enzyme activity. Likewise, positive cooperativity (h, 2.6) and apparent affinity to the cofactor (K_m, 22.0 µM) were almost identical to wild-type. But still, regulatory characteristics of PAH kinetics were altered with respect to a moderate increase in affinity to the substrate and a complete loss of substrate activation (Table 2). These findings point to a structural change of the V106A variant, which may have adopted a pre-activated 'r-state'-like conformation. Decreased *in vivo* enzyme activity (^{13}C-phenylalanine oxidation) was confirmed by *ex vivo* analyses (liver lysates) and in eukaryotic expression systems (COS-7 cells and TnT) (Fig. 1C). This was attended by a reduced amount of PAH protein in both the physiological context of the liver and the artificial expression system COS-7. These results suggested misfolding-induced proneness of V106A to degradation, potentially mediated by increased proteolytic turnover. Indeed, the presence of protease inhibitors led to an increase in the PAH protein amount in *Pah*^{enu1}, but not in wild-type liver lysates (Fig. 1D).

Table 2. Steady-state enzyme kinetic parameters of wild-type and variant murine PAH proteins

Genotype	Specific activity[a] (% of wild-type)	$[S]_{0.5}$[b] (µM)	K_m[c] (µM)	h[b]	Activation fold
WT	100	175 ± 34	22.0 ± 5	2.6	3.1
V106A	108 ± 14	103 ± 24	22.0 ± 6	2.6	1.0
F263S	4 ± 17	—	—	—	0.8

Recombinant tetrameric wild-type (WT) and variant (V106A, F263S) murine PAH proteins were expressed as MBP–PAH fusion proteins in *E. coli*. Apparent affinities for L-phenylalanine ($[S]_{0.5}$), BH_4 (K_m) and the Hill coefficient (h) as a measure of cooperativity are shown. Activation fold represents the ratio of specific activity with and without prior incubation with L-phenylalanine. Values are given as means ± s.e.m. of $n = 3$ experiments. The marked reduction in the specific PAH activity of F263S precluded the determination of further enzyme kinetic parameters.
[a]Specific activity was determined at standard L-phenylalanine (1 mM) and BH_4 (75 µM) concentrations with L-phenylalanine pre-incubation.
[b]Apparent affinities for L-phenylalanine ($[S]_{0.5}$) and the Hill coefficient (h) were determined at variable L-phenylalanine concentrations (0–4.5 mM) and standard BH_4 concentration (75 µM).
[c]Apparent affinities for BH_4 (K_m) were determined at variable BH_4 concentrations (0–700 µM) and standard L-phenylalanine concentration (1 mM).

Thus, loss of function in *Pah*^{enu1} is associated with reduced amounts of PAH protein and points to protein misfolding as the pathogenic trigger.

Misfolding induces PAH aggregation and degradation *in vitro*

The protein misfolding phenotype of *Pah*^{enu1} was analyzed by steady state, kinetic and thermal aggregation and degradation assays using recombinant protein. We monitored the oligomeric state of murine wild-type and V106A-PAH following affinity chromatography. Size-exclusion chromatography (SEC) revealed a similar tetramer-to-dimer ratio of both proteins, and this corresponded to human (4,27) and rat PAH (28), although V106A additionally showed a distinct peak of aggregates (Fig. 2A). Blue native polyacrylamide gel electrophoresis (BN-PAGE) showed tetramers, dimers and monomers in an apparent equilibrium for wild-type and variant PAH, with V106A displaying more high-molecular-weight aggregates and a higher amount of degradation fragments than the wild-type (Fig. 2B). Subsequent analyses focussed on the active enzyme homo-oligomer obtained by the separation of the tetrameric SEC fraction. The determination of particle sizes by dynamic light scattering (DLS) at the steady state revealed two populations for the wild-type corresponding to native-state protein and soluble aggregates in equilibrium. For V106A, a shift towards the non-native state was observed (Fig. 2C). Analysis of kinetics of soluble aggregate formation over time resulted in a significantly ($P = 0.0037$) higher exponential growth rate constant for V106A ($k = 0.072$) than for the wild-type ($k = 0.041$) (Fig. 2D).

Differential scanning fluorimetry (DSF) showed increased ground state hydrophobicity of V106A in comparison to the wild-type, indicating the presentation of non-native state hydrophobic groups at the protein surface. Thermal unfolding disclosed a two-phase transition from native to the unfolded state for wild-type and V106A. This corresponds to human PAH, where the first transition reflects unfolding of the regulatory and the second unfolding of the catalytic domain (4,29) (Fig. 2E). Although the shape and total height of the curves differed, midpoints of the first and second transitions were similar for wild-type and V106A.

The impact of local unfolding on protein stability as well as the equilibrium between native-state oligomeric protein and potential folding intermediates was further analyzed by limited proteolysis (30,31). Compared with the wild-type, V106A revealed degradation bands even without the addition of proteinase K and showed a different fragmentation pattern (Fig. 2F). The half-life of the intact PAH band (52 kDa) after the proteolytic cleavage of the MBP fusion partner was prolonged for V106A (34 min) in comparison to the wild-type (13 min). This finding may be explained by the fact that aggregates not attacked by proteinase K could appear as intact PAH in a denaturating SDS–PAGE.

Taken together, the equilibrium of native-state and partially unfolded or misfolded protein is shifted towards a distorted V106A conformation, explaining proneness to aggregation and susceptibility to degradation.

BH_4 rescues loss of function *in vivo*

We showed that *Pah*^{enu1} has the same pathophysiological background of protein misfolding with loss of function as that associated with human *PAH* mutations (4,14,16). We

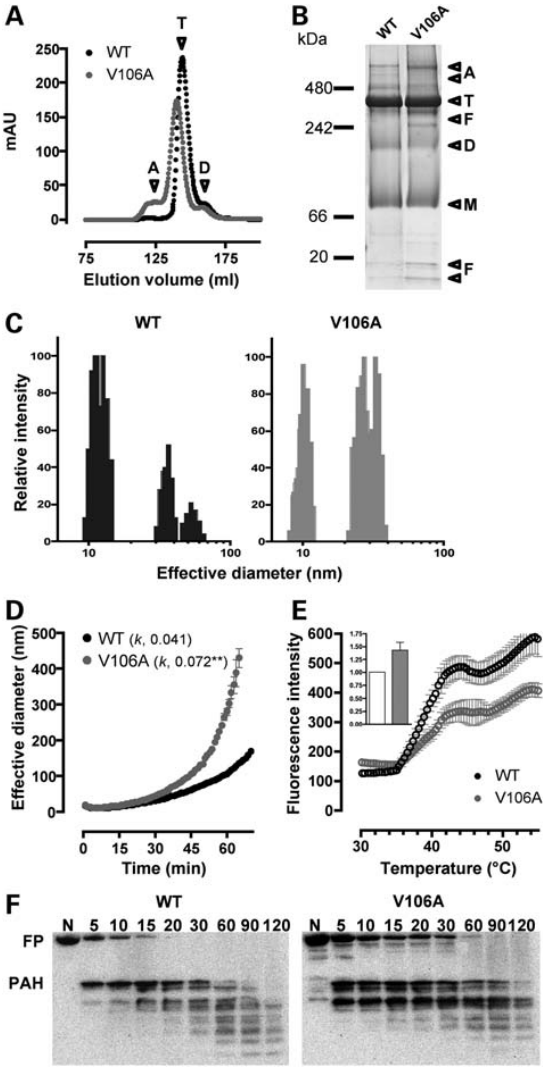

Figure 2. Impact of the V106A mutation on PAH protein conformation *in vitro*. Wild-type (WT) and V106A-PAH were expressed as fusion protein to MBP, purified by affinity chromatography and tetrameric MBP–PAH was separated by SEC. (**A**) Oligomerization profiles (normalized to tetrameric WT) determined by SEC. Arrows indicate soluble aggregates (A, 120 ml), tetramers (T, 140 ml) and dimers (D, 158 ml). (**B**) BN-PAGE analysis of affinity-purified protein. Arrows indicate aggregates (A), tetramers (T, 380 kDa), dimers (D, 190 kDa), monomers (M, 95 kDa) and degradation fragments (F). (**C**) Particle size distribution of the tetrameric SEC fraction determined by DLS. The cumulative relative occurrences (relative intensity, *y*-axis) of particles with distinct effective diameters (*x*-axis) of $n = 3$ independent experiments are shown. Expected diameter for tetrameric MBP–PAH is 16 nm. (**D**) Aggregation kinetics measured by DLS. The rate constants (k) of changes in the effective diameter of the protein are given and the significance is indicated ($^{**}P < 0.01$). Data points represent means ± s.e.m. of $n = 3$ experiments. (**E**) Thermal denaturation analyzed by DSF. Data points represent means ± s.e.m. of $n = 3$ experiments. The inset shows the fluorescence at 30°C normalized to WT. (**F**) Proteolytic degradation patterns of MBP–PAH fusion proteins. Samples were incubated without proteinase K (N) or for 5–120 min at 37°C with proteinase K. Fusion proteins (FP), the cleaved-off PAH protein (PAH) and proteolytic fragments of PAH were detected. One representative experiment out of four is shown for WT and V106A.

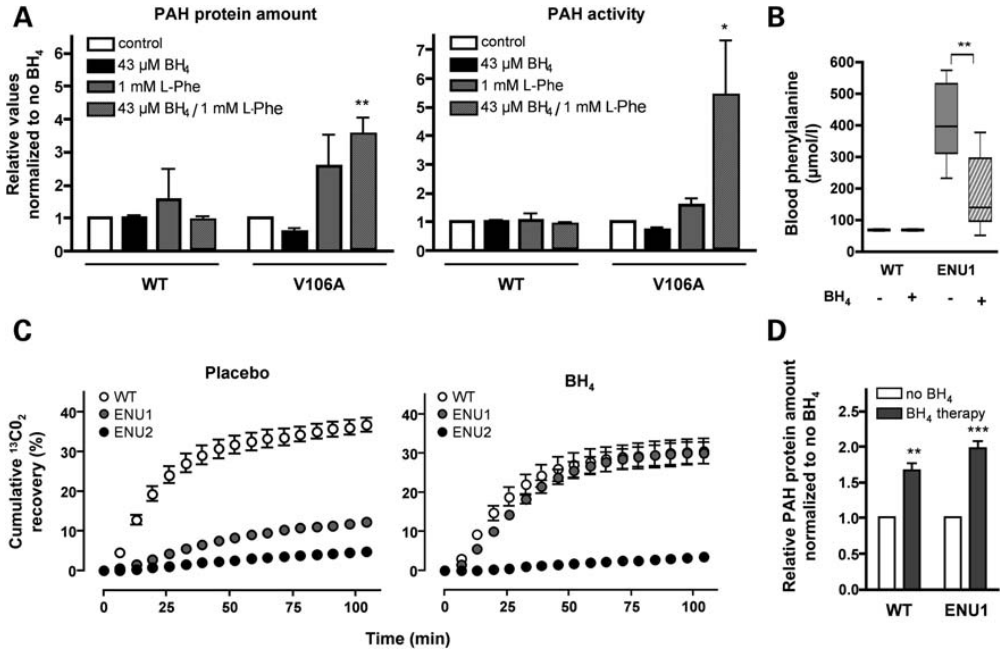

Figure 3. BH_4 stabilizes the PAH protein and corrects loss of function *in vivo*. (**A**) Wild-type (WT) and V106A-PAH proteins were overexpressed in COS-7 cells for 72 h and cultured in basic medium (RPMI 1640, L-phenylalanine 96 μM; control). The medium was supplemented with either BH_4 (43 μM), additional L-phenylalanine (final concentration 1 mM) or both BH_4 and L-phenylalanine as indicated. Data were normalized to the control and significances relative to the control are indicated ($^*P < 0.05$, $^{**}P < 0.01$). Bars represent means ± s.e.m. of $n = 5$ experiments. (**B**) Basic blood phenylalanine levels before and after BH_4 therapy. Wild-type (WT) and *Pah*^{enu1} (ENU1) mice were treated with either 20 μg BH_4/g bw or placebo for 3 days in two single doses per day. Box plots represent blood phenylalanine levels of $n = 4$ (WT) and $n = 7$ (ENU1) animals and the significance is indicated ($^{**}P < 0.01$). (**C**) Phenylalanine oxidation rates determined by a ^{13}C-phenylalanine breath test. Mice were treated with BH_4 (20 μg/g bw) or placebo and the cumulative CO_2 recovery was monitored in $n = 6$ animals for each genotype and is given as mean values ± s.e.m. (**D**) Relative amounts of PAH protein in liver lysates after treatment with BH_4 (20 μg/g bw for 3 days). Data were normalized to mice that received placebo and are given as means ± s.e.m. of $n = 3$ experiments. $^{**}P < 0.01$, $^{***}P < 0.001$.

now aimed to analyze whether the pharmacological chaperone BH_4 corrects these alterations. In our wild-type mice, one single dose of 10 μg/g body weight (bw), commonly used in the treatment of PAH-deficient patients, led to a liver peak concentration of 43 μmol/l BH_4 30 min after injection. Hence, this concentration was used for the following experiments in cell culture and *in vitro*.

The effect of BH_4 on PAH protein amount and enzyme activity was first characterized after transient expression of wild-type and V106A-PAH in COS-7 cells (Fig. 3A). Treatment with BH_4 at low phenylalanine concentrations (96 μmol/l in the medium) did not result in a rise of protein amount or enzyme activity in wild-type or V106A. Notably, pathologically high phenylalanine concentrations (1 mM) produced a mild increase in the amount of wild-type and, more pronounced, of the V106A-PAH protein. However, this had no perceptible effect on enzyme activity. Only the application of BH_4 at high phenylalanine concentrations resulted in a significant increase in V106A-PAH protein amount ($P < 0.01$) and activity ($P < 0.05$), suggesting that the pharmacological chaperone exerts its action predominantly at harmful substrate levels.

BH_4 treatment *in vivo* (2 × 10 μg/g bw × d, 3 days) significantly ($P < 0.01$) lowered blood phenylalanine concentrations (median, 396–139 μmol/l) in *Pah*^{enu1}, while the wild-type remained at physiological levels (median, 68 μmol/l) (Fig. 3B). The therapeutic effect of BH_4 on hyperphenylalaninemia was functionally confirmed by placebo-controlled ^{13}C-phenylalanine oxidation tests, demonstrating rescue of *in vivo* PAH activity. Treatment normalized $^{13}CO_2$ recovery in *Pah*^{enu1} to wild-type levels, whereas it had no effect in *Pah*^{enu2} (Fig. 3C). This was attended by a significant increase in liver PAH protein in the wild-type ($P < 0.01$) and, even more pronounced, in *Pah*^{enu1} ($P < 0.001$) (Fig. 3D).

These data show that BH_4 corrects the biochemical phenotype of *Pah*^{enu1} through stabilization of the PAH protein *in vivo*. Moreover, the pharmacological chaperone-mediated rescue of enzyme function requires an imbalanced metabolic state reflected by hyperphenylalaninemia and sufficient specific residual enzyme activity.

BH_4 corrects protein misfolding

To dissect the molecular mode of action of the pharmacological chaperone BH_4 at the protein level, we analyzed its effect on PAH misfolding/unfolding, aggregation and degradation. The addition of BH_4 (43 μM) significantly ($P < 0.01$) reduced the increased hydrophobicity of recombinant V106A, while it had no effect on the wild-type protein (Fig. 4A). Native-state stabilization with respect to the

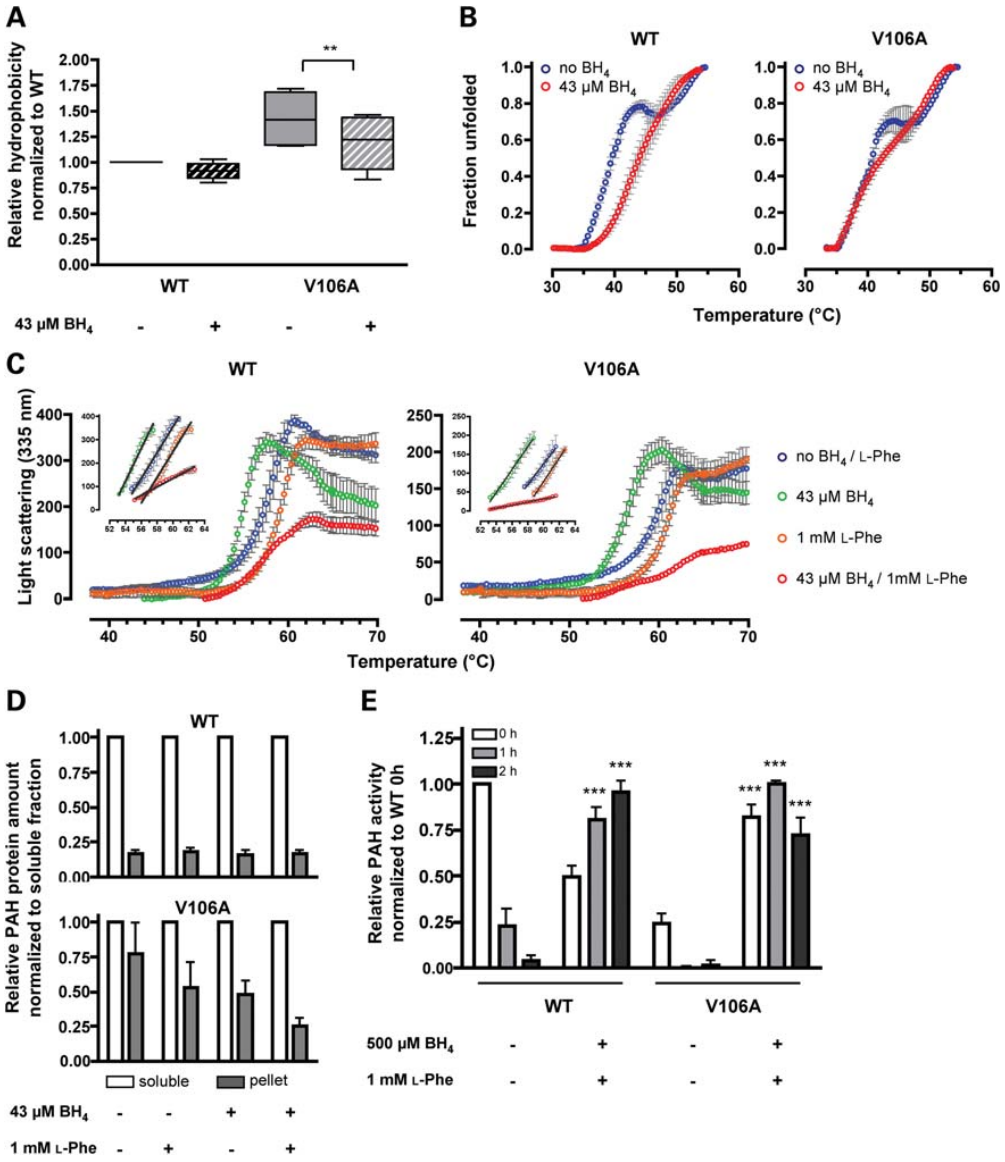

Figure 4. BH_4 prevents PAH aggregation and degradation. (**A**) Relative hydrophobicity of wild-type (WT) and V106A-PAH proteins determined by ANS fluorescence at 25°C with and without the addition of BH_4 (43 µM). Plots represent ANS-fluorescence values normalized to WT without BH_4 of $n = 4$ experiments and the significance is indicated (**$P < 0.01$). (**B**) Thermal unfolding of wild-type (WT) and V106A-PAH with and without the addition of BH_4 (43 µM) monitored by ANS fluorescence. The fractions of unfolded PAH protein are plotted as a function of increasing temperatures. Data points represent the mean ± s.e.m. of $n = 3$ experiments. (**C**) Temperature-induced aggregation of wild-type (WT) and V106A-PAH observed by RALS. Aggregate formation in the presence or absence of BH_4 (43 µM) and L-phenylalanine (1 mM) was detected. For the linear phase of aggregation, the transition midpoints and the slopes of the curves were calculated by linear regression analysis (Table 3). The insets depict the slopes at the linear phase of aggregation. Data points and calculated values represent means ± s.e.m. of $n = 3$ experiments. (**D**) Aggregation of WT and V106-PAH protein expressed in COS-7 cells upon treatment with BH_4 (43 µM) and/or L-phenylalanine (1 mM). The PAH amount in the soluble and pellet fractions was detected. Bars are normalized to the soluble fraction and represent means ± s.e.m. of $n = 4$ experiments. (**E**) Protection against degradation-induced loss of PAH function in a eukaryotic TnT system. Wild-type (WT) and V106A-PAH were expressed with or without the addition of BH_4 (500 µM) and L-phenylalanine (1 mM) as indicated. The reaction was stopped and the relative activity was assessed following further incubation for 0, 1 or 2 h at 37°C. Significances of changes in relation to the control (no BH_4/L-phenylalanine) are indicated (***$P < 0.001$). Data are normalized to wild-type activity at time point 0 and bars represent means ± s.e.m. of $n = 3$ experiments.

midpoint of unfolding (T_m) was observed upon application of thermal stress (wild-type, 38.96–44.15°C, $P = 0.042$; V106A, 40.39–42.01°C, $P = 0.184$). BH_4 induced a right shift of the low-temperature transition, indicating stabilization of the regulatory domain of the proteins, which was more pronounced in the wild-type (Fig. 4B). The minor effect for

Table 3. Parameters of aggregation kinetics assessed by RALS

	WT $T_{m1/2}$ (°C)	Slope	V106A $T_{m1/2}$ (°C)	Slope
No BH_4/L-Phe	57.20 ± 0.07	55.31 ± 3.46	58.95 ± 0.09	27.24 ± 4.07
43 μM BH_4	54.54 ± 0.03	69.44 ± 3.92	55.85 ± 0.06	30.19 ± 1.95
1 mM L-Phe	58.76 ± 0.05	54.54 ± 2.22	60.29 ± 0.06	31.64 ± 1.99
43 μM BH_4 / 1 mM L-Phe	57.14 ± 0.06	18.09 ± 0.68	59.09 ± 0.10	4.00 ± 0.17

Temperature induced aggregation of wild-type (WT) and V106A-PAH observed by RALS. Aggregate formation in the presence or absence of 43 μM BH4 and 1 mM L-phenylalanine was detected. Transition midpoints ($T_{m1/2}$) defined as the temperature at half-maximum aggregation were calculated by nonlinear regression analysis using the Boltzman sigmoidal equation. For the linear phase of aggregation, the slopes of the curves were calculated by linear regression analysis. Values represent means ± s.e.m. of $n = 3$ experiments.

V106A may be attributed to a conformational rearrangement of the regulatory domain due to the variant enzyme's pre-activation (Table 2).

Protein aggregate formation upon thermal stress was assessed by right angle light scattering (RALS). Without additives, aggregation occurred in the range between 50 and 60°C and reached a plateau at 60°C (Fig. 4C). Addition of the cofactor BH_4 led to aggregation at lower temperatures, whereas pathological concentrations of the substrate L-phenylalanine (1 mM) led to aggregation at higher temperatures for wild-type and V106A (Table 3). Neither phenylalanine nor BH_4, however, markedly changed the slope of the curves when applied alone. Only the combined application of BH_4 and phenylalanine substantially reduced aggregation rates, with a more pronounced effect in V106A (slope reduced to 14.7% of initial) than in the wild-type (32.7% of initial).

These results were substantiated at the cellular level. In transiently transfected COS-7 cells, wild-type PAH was predominantly found in the soluble fraction with <20% of the protein in the pellet (Fig. 4D). V106A, though, accumulated in the pellet fraction, indicating enhanced aggregation *in vivo*. Supplementation of the medium with BH_4 or phenylalanine (1 mM) moderately reduced variant PAH in the pellet. However, a combined application of BH_4 and phenylalanine markedly decreased aggregation. We then investigated the impact of the pharmacological chaperone BH_4 on PAH activity over time in a eukaryotic TnT system (Fig. 4E). In the absence of BH_4 and phenylalanine, wild-type and V106A activity declined within 1 h, where the initial activity of V106A was lower than that of the wild-type and decreased more rapidly. In the presence of BH_4 and phenylalanine, loss of wild-type and V106A activity was reverted and V106A reached wild-type levels. The initial reduction of wild-type activity upon addition of BH_4 is in line with the inhibitory effect of BH_4 observed in the phenylalanine oxidation tests (Fig. 3C) and reflects the well-known cofactor inhibition of PAH activity (32–34).

In conclusion, BH_4 exerts its effect as a pharmacological chaperone in the *Pah*^{enu1} mouse by stabilization of PAH conformation and thus preventing aggregation and protecting the mutant enzyme from rapid degradation.

DISCUSSION

The relevance of protein misfolding for inherited diseases is increasingly appreciated and novel therapeutic strategies emerge. A substantial number of small-molecule chemical compounds, which either stabilize the distorted protein conformation or restore the function of affected biological pathways, were lately identified (Supplementary Material, Table S1) (7,9–11). One of these is BH_4, recently approved by the FDA and the EMEA as an orphan drug to correct protein misfolding in PAH deficiency. This makes BH_4 the first representative of a new class of pharmaceutical drugs, and further stabilizing compounds for other diseases are likely to follow.

Here, we report that the *Pah*^{enu1} mouse shows BH_4-responsive PAH deficiency and therefore is a model for a treatable protein misfolding disease with loss of function. Although *Pah*^{enu1} displays a biochemical phenotype, the specific PAH activity is normal. We identified loss of PAH protein as a cause of loss of function in this animal model. It is a consequence of misfolding and accelerated degradation of the enzyme. Notably, our *in vitro* and *in vivo* data revealed that aggregation also significantly contributes to loss of function. The elucidation of this triad created the basis to dissect the molecular mode of action of the pharmacological chaperone BH_4 bridging *in vitro* data on protein stabilization to *in vivo* metabolic state and enzyme activity.

The PAH protein is a flexible, highly regulated tetrameric enzyme undergoing conformational changes upon binding of the L-phenylalanine substrate and the natural cofactor BH_4 (35–37). The latter functions as a negative effector to form a dead-end PAH–BH_4 complex *in vivo*. This keeps the enzyme in an inactivated form that is activated by high blood phenylalanine concentrations (38). As previously described for human mutations (4,16), the genetic alteration in this mouse does not lead to changes in PAH affinity to the cofactor or to a marked reduction of specific enzyme activity. Thus, the mode of action of BH_4 in PAH deficiency is not related to its cofactor action. Instead, pharmacological doses of BH_4 lead to protein stabilization augmenting the effective PAH concentration, that is, the amount of correctly folded and functional PAH. This is achieved by decelerating aggregation, reducing protein hydrophobicity and slowing-down degradation. At a structural-mechanistic level, the improved biochemical phenotype is the result of a trade-off between a gain in protein rigidity and a loss of enzyme activity. Whereas wild-type mice display a drug-induced decrease in PAH activity, in *Pah*^{enu1} the increase in effective enzyme concentration overrules the inhibition by BH_4 and thus explains complete correction of enzyme activity *in vivo*. Hence, the chemical compound has a direct influence on PAH protein chemistry, shifting the activated, instable and aggregation-prone conformation towards a less active but stabilized state.

We observed that BH_4 was very effective at high phenylalanine concentrations, whereas it did not increase enzyme activity at low substrate levels. This phenomenon of selective pharmaceutical action adjusted to metabolic needs can be ascribed to the complex regulation of the phenylalanine hydroxylating system known from human and rat PAH (32–34,39), and here confirmed to be analogous in the mouse. The physio-

logical inhibitory action of the cofactor BH_4 is reflected in the pharmacological inoperativeness of the drug at low substrate concentrations. This is of particular importance since it prevents undue elimination of the essential amino acid phenylalanine and thus protects the patient from overtreatment. This makes BH_4 a pharmacological chaperone drug with the ideal characteristic of an integrated self-limiting mechanism considerably improving its safety profile.

The animal model described here will allow for studies leading to significant advances for patients with PKU. It will enable further pharmacological studies that address detailed pharmacodynamics and pharmacokinetics of BH_4 in a mouse displaying the disease-specific phenotype, and it will provide valuable information towards individually tailored treatment strategies by its use to explore BH_4 dosing at various metabolic states. Moreover, an *in vivo* model will be mandatory for the development of optimized BH_4 derivatives and future generations of PAH stabilizing compounds (40) with improved bioavailability and efficacy.

However, the use of *Pah*^{enu1} will not be confined to issues related to PAH. Direct analyses of the misfolded protein's function (*in vivo* enzyme activity) and the biochemical phenotype (metabolite concentrations) in the living animal offer the opportunity to investigate universal cellular pathways modulating loss-of-function pathology. These will include the concerted action of molecular chaperones, the unfolded protein response pathway, and the protein degradation machinery. Moreover, the impact of epigenetic and environmental stress factors, such as single nucleotide polymorphisms, oxidative stress, or fever, which trigger or exacerbate conformational diseases, can be analyzed. Finally, the model may be used to explore the efficacy of alternative compounds or combinatorial therapeutic strategies, where pharmacological chaperones act in synergy with antioxidative substances, proteasome inhibitors or regulators of protein homeostasis (7,11).

In conclusion, our data support a general model of pharmacological chaperone-induced stabilization of protein conformation to correct protein misfolding with loss of function. The knowledge gained and the experimental strategies applied will be applicable to other maladies belonging to this group of disorders and may thus promote the development of new drugs for the treatment of misfolding-associated genetic and non-genetic diseases.

MATERIALS AND METHODS

Plasmid constructs and site-directed mutagenesis

The cDNA of murine *Pah* (EST clone from imaGenes) was cloned into the pcDNA3.1/myc-His C vector (Invitrogen). The mutations V106A and F263S were introduced into the pcDNA3.1 plasmid using the PCR-based QuikChange site-directed mutagenesis kit (Stratagene) and authenticity was verified by DNA sequencing. Subsequently, wild-type and mutant cDNAs were subcloned into the prokaryotic expression vector pMAL-c2E (New England Biolabs) or the eukaryotic expression vector pEF-DEST51 (Invitrogen). In order to create constructs for eukaryotic expression of wild-type and variant PAH, the Gateway technology (Invitrogen) was used. Genes were amplified using primers containing flanking *att*B sites, Kozak sequence at the 5′ terminus (Mm*Pah* forward) and a stop codon at the 3′ terminus (Mm*Pah* reverse) (Supplementary Material, Table S2). Obtained PCR products were subsequently cloned into the pEF-DEST51 vector carrying the human elongation factor promotor (Invitrogen), following the manufacturer's protocols.

Protein expression and purification

Expression plasmids were transformed into *E. coli* DH5α. Wild-type and variant fusion proteins (MBP–PAH) were overexpressed for 20 h at 37°C as described previously (4). Proteins were purified on ÄKTAxpress (GE Healthcare) at 4°C by loading crude extracts on a MBPTrap affinity chromatography column (GE Healthcare) equilibrated with column buffer (20 mM Tris–HCl, 200 mM NaCl, pH 7.4) and by elution with the same buffer supplemented with 10 mM maltose.

PAH activity assay

PAH activity was determined as described previously (27,41) with various modifications (4). Recombinant tetrameric PAH (0.01 mg/ml) was pre-incubated with L-Phe and catalase (1 mg/ml) for 5 min (25°C) in 15 mM Na HEPES, pH 7.3, followed by 1 min incubation with 10 μM ferrous ammonium sulfate. The reaction was initiated by the addition of 6[R]-L-erythro-5,6,7,8-tetrahydrobiopterin (BH_4, Schircks Laboratories) stabilized in 2 mM DTT, carried out for 1 min and stopped by acetic acid, followed by 10 min incubation at 95°C. Kinetic parameters were determined at standard L-Phe (1 mM) and BH_4 (75 μM) concentrations at variable BH_4 (0–704.1 μM) or L-Phe (0–4.5 mM) concentrations, respectively. To determine the level of substrate activation, pre-incubation with the substrate was omitted and the reaction was triggered by simultaneous addition of 1 mM L-Phe and 75 μM BH_4 (activation fold). All concentrations mentioned refer to the final concentration in a 100 μl reaction mixture. The amount of the product (L-tyrosine) was measured by HPLC and assayed as triplicates. In the case of PAH obtained from either cell culture or the TnT system, 20 μl of total lysates were used for the activity assay. The reaction initiated by the addition of BH_4 was carried out for 60 min (cell culture and TnT) or 20 min (liver lysates), respectively. Steady-state kinetic parameters of three independent experiments were calculated by nonlinear regression analysis with the use of GraphPad Prism 4.0c (GraphPad Software). Enzyme kinetic parameters at variable substrate concentrations displayed cooperativity and were calculated with the Hill equation. Enzyme kinetic parameters at variable cofactor concentrations were calculated with the Michaelis–Menten equation. All experimental data were confirmed by repeated analyses of different protein purifications.

Analysis of oligomerization

Oligomerization profiles were obtained by SEC on a HiLoad 26/60 Superdex 200 column (GE Healthcare) equilibrated with SEC buffer (20 mM Na HEPES, 200 mM NaCl, pH 7.0) and relative amounts of the different oligomeric states were

calculated as described previously (4). Protein concentrations were determined spectrophotometrically with the use of the absorption coefficient A_{280} or the Bradford assay. All following experiments using recombinant protein, except for the BN-PAGE analysis, were performed with tetrameric fusion proteins.

Blue native polyacrylamide gel electrophoresis

BN-PAGE was used to analyze non-dissociated protein complexes with respect to composition, oligomeric state and molecular mass. For this purpose, the protein pool of affinity-purified wild-type mouse PAH was separated via native PAGE using 4–16% (w/v) pre-cast polyacrylamide-gradient native gels in a Bis–Tris buffer system (Invitrogen). The electrophoresis was started at 150 V for 60 min and continued for 90 min at 250 V at 4°C. Following BN-PAGE, proteins were denatured in the gel, stained with Coomassie Brilliant Blue R-250 (Bio-Rad) and the oligomeric state was determined in comparison to protein standards (GE Healthcare).

SDS–PAGE and immunoblotting

The proteins were separated by 4–12% gradient polyacrylamide gels (Invitrogen) and blotted onto a nitrocellulose membrane (Schleicher & Schuell BA-S 85). The membrane was blocked with 5% milk in 1× TBS followed by 1 h of incubation with the primary antibody, mouse monoclonal anti-PAH PH8 (Calbiochem, 1 : 1000 dilution), and 1 h of incubation with the secondary antibody, anti-mouse IgG conjugated with alkaline phosphatase (Promega, 1 : 7500 dilution). Blots were visualized with CDP-Star substrate (Roche) and chemiluminescence was monitored with a DIANA III imaging system, and the resulting protein bands were quantified by AIDA software (Raytest).

Dynamic light scattering

DLS analysis was performed on a ZetaPALS particle size analyzer (Brookhaven Instruments Corporation) equipped with a precision Peltier temperature control unit. The steady-state determination of particle sizes of the tetrameric fusion proteins MBP–PAH (10 μM PAH subunit) diluted in SEC buffer was performed at 25 °C at time intervals of 1 min over a period of 10 min in six independent measurements. The kinetics of thermal aggregation over time was monitored at 52°C at time intervals of 1 min until the precipitation of insoluble aggregates. Analysis of three independent experiments was performed by fitting the obtained data to a single exponential growth curve. The scattering angle was 90°.

Limited proteolysis by proteinase K

Experiments were performed as described previously (4). Wild-type and V106A murine MBP–PAH were digested with proteinase K (substrate to protease ratio 1 : 5000) at 37°C in SEC buffer. SDS–PAGE electrophoresis and immunoblotting with anti-PAH antibody were performed and the resulting PAH bands were quantified by AIDA software (Raytest).

Thermal denaturation assays

RALS and DSF analyses were performed on a Cary Eclipse fluorescence spectrophotometer equipped with a temperature-controlled Peltier multicell holder (Varian). Denaturation of protein samples diluted in SEC buffer containing 1 mM DTT was performed by scanning a temperature range of 25–85°C at a rate of 1.2°C/min (25–35°C and 75–85°C) or 0.2°C/min (35–75°C). In the cases indicated, L-phenylalanine (Sigma) and BH_4 were added to final concentrations of 1 mM and 43 μM, respectively. In DSF experiments, the changes in 8-anilino-1-naphtalenesulfonic acid (ANS, Sigma Aldrich) fluorescence emission at 500 nm (excitation 395 nm, slit widths 5.0/10.0 nm) were monitored. Data analysis was performed by fitting the experimental curves as described previously (40). The phase transitions of three independent experiments were determined and the respective transition midpoints were calculated by differentiation of the increasing part of the curves. We previously showed that the denaturation of MBP did not interfere with the unfolding curves of the PAH protein for ANS fluorescence (4). Protein aggregation based on hydrophobic self-association was determined by RALS. The intensity of scattered light at 335 nm was detected in the right angle to the excitation light of 330 nm upon thermal denaturation of MBP–PAH (0.8 μM PAH subunit). Data points were analyzed by the Boltzman sigmoidal equation for the transition temperatures and the first-order straight-line exponential equation for the slope.

Coupled *in vitro* transcription–translation of PAH

In vitro transcription and translation (TnT) of mouse PAH was carried out using the pcDNA3.1 constructs and the TnT-T7 reticulocyte lysate system (Promega). The TnT reaction was performed for 90 min in the absence or presence of 500 μM BH_4 and 1 mM L-Phe in 2 mM DTT. After stopping the reaction by DNase A and RNase A, the lysates were further incubated at 37°C and aliquots were taken at indicated time points. For PAH activity assays, free amino acids were removed by microcon centrifugal filters (Millipore).

Transient expression of PAH in COS-7

COS-7 cells were maintained in basic RPMI 1640 medium (96 μM L-phenylalanine) with stable glutamine supplemented with 10% fetal bovine serum and 1% antibiotics (PAA). For transient expression of wild-type and V106A-PAH, the pEF-DEST51 cDNA constructs were used. After transfection with 3 μg DNA per 1 million cells using the Amaxa electroporation system (Lonza), cells were cultured for 72 h under three different conditions: (i) basic medium (as described above), (ii) basic medium supplemented with 43 μM BH_4 and 5 μg/ml ascorbic acid (Fluka), (iii) basic medium with 43 μM BH_4, 5 μg/ml ascorbic acid and 1 mM L-Phe. Culture medium was changed every 24 h. Cells were harvested by scraping and lysed by three freeze–thaw cycles in a lysis buffer (SEC buffer containing 1% Triton X-100 and proteinase inhibitors), followed by 20 min centrifugation at 14 000 rpm at 4°C. Both recovered supernatants and pellets resuspended

in lysis buffer were kept at −80°C until being used for SDS–PAGE/immunoblotting and activity assays.

In vivo animal studies

Wild-type (BTBR $T^{+}tf$/J), *Pah*^{enu1} (BTBR.Cg-*Pah*^{enu1}/J) and *Pah*^{enu2} (BTBR-*Pah*^{enu2}/J) mice were purchased from The Jackson Laboratory and kept under standard conditions. Mouse studies were approved by the Austrian Animal Care and Use Committee in accordance with national and international laws and standards for animal protection. Animals were housed in a controlled temperature room maintained under alternating 12 h light and dark cycles and, in between experiments, had free access to food and water. While *Pah*^{enu1} and wild-type mice were fed with standard breeder mouse chow (UIZ, Knittelfeld, Austria), *Pah*^{enu2} mice were kept euphenylalaninemic with a diet devoid of phenylalanine (Teklad 97152) and water containing 30 mg L-phenylalanine. They were placed on standard chow 3 days before the experiments to produce a hyperphenylalaninemic state. The effect of BH_4 (20 μg/g bw b.i.d. by intraperitoneal injection for 3 days) or placebo (NaCl 0.9%, ascorbic acid 0.2%) was assessed on blood phenylalanine concentrations and on *in vivo* phenylalanine oxidation. For this purpose, breath tests were performed in conscious mice. After the i.p. application of ^{13}C-labeled L-phenylalanine (15 μg/g bw, Eurisotop), the mice were placed in individual 100 ml breath chambers with a continuous flow of CO_2-free air for 105 min and breath samples were collected in 6.5 min intervals. For each of the samples, the airflow was discontinued to allow CO_2 levels to accumulate above the limit of quantification (0.8%). At the end of accumulation, the sample was insufflated into an infrared spectrometer (IRIS Wagner Analysen Technik, Germany) for online isotope analysis. The cumulative recovery of ^{13}C in breath samples was calculated based on the $^{13}CO_2/^{12}CO_2$ ratio assuming a total CO_2 production rate of 94 ml/min per g bw × m^2 body surface area. The amount of labeled CO_2 formed was expressed as the cumulative percentage of the dose administered as a function of time. Blood samples for the determination of blood phenylalanine concentrations were collected immediately before the breath test and spotted to filter cards. Then, 3.2 mm spots of these samples were extracted with 200 μl of methanol (containing internal standard) and analyzed by electrospray ionization tandem mass spectrometry. For assessment of PAH activity and PAH protein amount, mice were sacrificed by cervical dislocation 1 h after the last dosage. Liver samples were collected and processed immediately. The liver tissue was homogenized and lysed in a buffer containing 200 mM KCl, 30 mM Tris–HCl, pH 7.25, and proteinase inhibitors. Samples were then centrifuged for 50 min at 4°C and 14 000 rpm and resulting supernatants were frozen in liquid nitrogen until being used for SDS–PAGE/immunoblotting and activity assays. Protein concentration was determined using the Bradford assay.

Statistical analyses

Group mean values were compared by Student's unpaired two-tailed *t* test, and one-way analysis of variance with Dunnett's *post hoc* test was applied for multiple comparisons. Statistical analyses were performed using GraphPad Prism 4.0c (GraphPad Software).

SUPPLEMENTARY MATERIAL

Supplementary Material is available at *HMG* online.

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Conflict of Interest statement. None declared.

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New insights into tetrahydrobiopterin pharmacodynamics from *Pah*$^{enu1/2}$, a mouse model for compound heterozygous tetrahydrobiopterin-responsive phenylalanine hydroxylase deficiency

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ABSTRACT

Phenylketonuria (PKU), an autosomal recessive disease with phenylalanine hydroxylase (PAH) deficiency, was recently shown to be a protein misfolding disease with loss-of-function. It can be treated by oral application of the natural PAH cofactor tetrahydrobiopterin (BH_4) that acts as a pharmacological chaperone and rescues enzyme function *in vivo*. Here we identified *Pah*$^{enu1/2}$ bearing a mild and a severe mutation (V106A/F363S) as a new mouse model for compound heterozygous mild PKU. Although BH_4 treatment has become established in clinical routine, there is substantial lack of knowledge with regard to BH_4 pharmacodynamics and the effect of the genotype on the response to treatment with the natural cofactor. To address these questions we applied an elaborate methodological setup analyzing: (i) blood phenylalanine elimination, (ii) blood phenylalanine/tyrosine ratios, and (iii) kinetics of *in vivo* phenylalanine oxidation using ^{13}C-phenylalanine breath tests. We compared pharmacodynamics in wild-type, *Pah*$^{enu1/1}$, and *Pah*$^{enu1/2}$ mice and observed crucial differences in terms of effect size as well as effect kinetics and dose response. Results from *in vivo* experiments were substantiated *in vitro* after overexpression of wild-type, V106A, and F263S in COS-7 cells. Pharmacokinetics did not differ between *Pah*$^{enu1/1}$ and *Pah*$^{enu1/2}$ indicating that the differences in pharmacodynamics were not induced by divergent pharmacokinetic behavior of BH_4. In conclusion, our findings show a significant impact of the genotype on the response to BH_4 in PAH deficient mice. This may lead to important consequences concerning the diagnostic and therapeutic management of patients with PAH deficiency underscoring the need for individualized procedures addressing pharmacodynamic aspects.

1. Introduction

Phenylketonuria (PKU; [MIM 261600]) is an autosomal recessive inborn error of metabolism caused by deficiency of hepatic phenylalanine-4-hydroxylase (PAH; EC 1.14.16.1). PAH catalyzes the rate-limiting step in phenylalanine catabolism and is regulated by binding of its L-phenylalanine substrate and the cofactor 6*R*-L-*erythro*-5,6,7,8-tetrahydrobiopterin (BH_4) and by phosphorylation [1–3]. Mutations in the *PAH* gene lead to loss-of-function of the PAH protein, which is often induced by protein misfolding [4]. The clinical phenotypes of hyperphenylalaninemia due to PAH deficiency are classified from classic PKU (blood phenylalanine concentrations > 1200 μM) to mild PKU (600–1200 μM) and mild hyperphenylalaninemia (MHP, 120–600 μM). Patients with classic and mild PKU need lifelong treatment to prevent mental retardation, whereas the necessity of MHP treatment is under

Abbreviations: PKU, phenylketonuria; PAH, phenylalanine hydroxylase; BH_4, 6*R*-L-*erythro*-5,6,7,8-tetrahydrobiopterin; MIM, Mendelian inheritance in man number; EC, Enzyme Commission number; MHP, mild hyperphenylalaninemia; enu, N-ethyl-N-nitrosourea; BTBR, black and tan brachyuric mouse strain; DTT, dithiothreitol; K_e, elimination constant; c_0, initial concentration; c_{max}, peak concentration; t_{max}, time to peak concentration; AUC, area under the concentration vs. time curve at 0–180 min; $t_{1/2}$, elimination half-life; ^{13}C-phenylalanine, L-[1-13]C-phenylalanine; DOB, delta over baseline; DOB_{BH4}, delta over baseline after BH_4 treatment; $DOB_{Placebo}$, delta over baseline after Placebo; Phe_0, initial phenylalanine concentration; Phe_{180}, phenylalanine concentration at 180 min.

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debate [5,6]. In the last 60 years the only treatment available for patients with PAH deficiency was dietary phenylalanine restriction, a burdensome treatment associated with significant risks of malnutrition. A paradigm change occurred 10 years ago when researchers observed that pharmacological doses of the natural PAH cofactor, BH_4, can reduce blood phenylalanine concentrations in a significant number of patients that do not display one of the rare forms of BH_4 deficiency [7–9]. This led to definition of a new clinical phenotype, BH_4-responsive PAH deficiency. Efficacy and safety of sapropterin dihydrochloride, the synthetic form of BH_4, was subsequently demonstrated in clinical trials [10–13] and the Food and Drug Administration (FDA) and the European Medicines Agency (EMEA) approved Kuvan® (Merck Serono) as an orphan drug to treat BH_4-responsive PAH deficiency. However, at the time of market approval the molecular mode of action of the new drug was not well understood. By now, analyses of the pharmacological BH_4 effect *in vitro* performed by different groups have provided evidence for structural stabilization of misfolded PAH indicating a pharmacological chaperone mode of action of BH_4 [14–16].

In addition, an animal model with the specific clinical and biochemical phenotype of BH_4-responsive PAH deficiency was not available for drug approval. PAH deficiency in mice has previously been generated by germline mutagenesis [17,18]. The V106A mutation in *Pah*$^{enu1/1}$ leads to a MHP phenotype [17], whereas *Pah*$^{enu2/2}$, harboring the null-mutation F263S, shows classical PKU (www.pahdb.mcgill.ca) [18–20]. We recently showed that mice homozygous for the V106A mutation display a molecular phenotype of protein misfolding with loss-of-function and that *Pah*$^{enu1/1}$ is an animal model for the clinical phenotype of BH_4-responsive MHP [16]. However, patients that most benefit from treatment with BH_4 are those with mild PKU [8,21,22]. Moreover, the majority of patients (87%) with BH_4-responsive PAH deficiency are compound heterozygous carrying two different mutations on the maternal and the paternal allele [23] with about 26% of the alleles being putative null-mutations. Recent studies showed a marked influence of the patient's genotype on the intensity and time frame of response to BH_4 [24–26].

Previous clinical studies mostly analyzed the effect of BH_4 on the patients' blood phenylalanine level, an endpoint with limited functional value. We showed that the effect of BH_4 on blood phenylalanine concentrations in humans is accompanied by an increase in PAH function *in vivo* [8] and recently replicated this in the mouse model *Pah*$^{enu1/1}$ [16]. However, comprehensive pharmacodynamic and pharmacokinetic studies in a specific mouse model displaying the clinical phenotype of BH_4-responsive PAH deficiency are still not available.

Thus, the aims of this study were: (i) to investigate whether the compound heterozygous strain *Pah*$^{enu1/2}$ is a model for BH_4-responsiveness in mild PKU and (ii) to characterize pharmacodynamics and pharmacokinetics of BH_4 treatment in the mouse models *Pah*$^{enu1/1}$ and *Pah*$^{enu1/2}$. Our results show that *Pah*$^{enu1/2}$ is a model for compound heterozygous mild PKU with BH_4-responsiveness. Pharmacodynamics of BH_4, in particular with respect to effect size and effect kinetics differed significantly in both strains, while pharmacokinetics was congruent.

2. Materials and methods

2.1. Animals

BTBR, *Pah*$^{enu1/1}$ and *Pah*$^{enu2/2}$ mice were purchased from Jackson Laboratory (Bar Harbor, USA). The compound heterozygous hybrids *Pah*$^{enu1/2}$ were crossbred in our animals' facility. The animals were housed under controlled temperature conditions and maintained on a cycle of 12 h light/dark period. Between experiments water and food were available *ad libitum*. Basic blood phenylalanine concentrations and response to a single dose of BH_4 20 μg/g body weight (bw) without phenylalanine load were assessed after 1 h fasting from food to minimize postprandial phenylalanine fluctuations. All blood samples were taken from the tail and collected on filter cards. All animal experiments were approved by the Austrian Ministry of Science. Tests were carried out in adult animals at 3–6 months of age.

2.2. Combined phenylalanine-BH_4-loading tests and ^{13}C-phenylalanine breath tests

In order to minimize the influence of naturally occurring ^{13}C from food and to standardize phenylalanine supply, the experiments were performed after an overnight deprivation of food, whereas free access to water was allowed.

2.2.1. Combined phenylalanine-BH_4-loading test

Unlabelled L-phenylalanine (Sigma–Aldrich, St. Louis, USA) 15 μg/g bw and BH_4 (Cayman Chemicals, Tallin, Estonia) 20 μg/g bw or placebo (sodium chloride 0.9%, ascorbic acid 1%) were simultaneously injected intraperitoneally (i.p.). The compounds were dissolved in a sodium chloride 0.9% (B. Braun, Melsungen, Germany), ascorbic acid (Merck, Darmstadt, Germany) 1% solution at concentrations of 3 μg/ml (L-phenylalanine) and 4 μg/ml (BH_4) resulting in an injection volume of 5 μl/g. Venous blood samples of approximately 40 μl were collected on filter cards before and 20, 40, 60, 120, and 180 min after injection. Blood phenylalanine and tyrosine were quantified by electron spray ionization-tandem mass spectrometry. Blood phenylalanine elimination was analyzed by non-linear curve fitting of the concentrations at 20–180 min using a single exponential function:

$$Y = (Y_0 - \text{Plateau}) \times (e^{-K \times X}) + \text{Plateau}$$

2.2.2. ^{13}C-phenylalanine breath tests

L-[1-13]C-phenylalanine (^{13}C-phenylalanine, Eurisotop, Saint-Aubin Cedex, France) was injected in a dose of 15 μg/g bw i.p. simultaneously with BH_4 or placebo. Preparation and application of the compounds were performed as described above. Mice were placed in individual 100 ml breath chambers with a continuous flow of CO_2-free air. Breath samples were collected in 6.5-min intervals over a 104-min period. For each sample the airflow was discontinued for 90 s to allow CO_2 levels to accumulate above 0.8%. At the end of accumulation the sample was insufflated into an infrared spectrometer (IRIS Wagner Analysen Technik, Bremen, Germany) for online isotope analysis. The cumulative recovery of ^{13}C was calculated based on the ratio of $^{13}CO_2$ to $^{12}CO_2$ as previously described [27], assuming a total CO_2 production rate of 94 ml per min per g bw × m^2 body surface area [28]. For dose response studies, BH_4 was given in doses of 5, 10, 20, 30, and 40 μg/g bw, respectively. To analyze the effect duration, 40 μg/g bw of BH_4 were given 90, 24, 18, 9, 6, 4.5 or 3 h before or simultaneously to ^{13}C-phenylalanine. Subsequently, the cumulative recovery of $^{13}CO_2$ was determined at 104 min.

2.3. BH_4 pharmacokinetics

In order to determine pharmacokinetics, BH_4 at a dose of 20 μg/g bw was prepared and injected as described above. Venous blood samples (approximately 40 μl) were collected on filter cards before and 20, 40, 60, 120, and 180 min after injection. Total biopterin (BH_4, dihydrobiopterin, and biopterin) was quantified by High Pressure Liquid Chromatography (HPLC) as previously described [29,30]. Biopterin clearance was determined by non-

linear regression of a double exponential Bateman function as described by Koch et al. [31].

2.4. Transient expression of PAH in COS-7

COS-7 cells were maintained in basic RPMI 1640 medium (PAA Laboratories, Pasching, Austria) with stable glutamine supplemented with 10% fetal bovine serum (PAA) and 1% antibiotics (Antibiotic–Antimycotic; PAA). For transient expression of the murine wild-type and variant PAH pEF-DEST51 (Invitrogen, San Diego, USA) cDNA constructs coding for wild-type, V106A, and F263S PAH were used in single transfection (wild-type, $Pah^{enu1/1}$, $Pah^{enu2/2}$) or co-transfection ($Pah^{enu1/2}$). A total amount of 3 μg DNA per 1 million cells was applied using the Amaxa electroporation system (Lonza, Basel, Switzerland). Cells were cultured for 24 and 72 h under two different conditions: (i) basic medium (as described above), (ii) basic medium with 43 μM BH_4, 5 μg/ml ascorbic acid, and 1 mM L-phenylalanine. Culture medium was changed every 24 h. The cells were harvested and lysed by three freeze–thaw cycles in a lysis buffer containing 1% Triton X-100 and proteinase inhibitors, followed by 20 min centrifugation at 14,000 rpm, 4 °C. Recovered supernatants were subsequently used for activity assays [4].

2.5. PAH activity assay

PAH enzyme activity was determined as previously described [4,32,33] with modifications. 20 μl of total lysates obtained from cell culture were preincubated with 1 mM L-phenylalanine and catalase 1 mg/ml (Sigma–Aldrich, St. Louis, USA) for 5 min (25 °C) in 15 mM Na HEPES pH 7.3, followed by 1 min incubation with 10 μM ferrous ammonium sulphate (Sigma–Aldrich). The reaction was initiated by the addition of 75 μM BH_4 stabilized in 2 mM dithiothreitol (DTT; Fluka Chemie AG, Buchs, Switzerland), carried out for 60 min at 25 °C and stopped by acetic acid followed by 10 min incubation at 95 °C. All concentrations mentioned refer to the final concentration in a 100 μl reaction mixture. The amount of L-tyrosine production was measured and quantified by HPLC, assayed as triplicates. Three independent experiments were performed.

2.6. Statistics

Group mean values were compared by Student's unpaired two-tailed *t*-test. Statistical analyses were performed using GraphPad Prism 4.0c (GraphPad Software, San Diego, USA).

3. Results

3.1. $Pah^{enu1/2}$ is a model for compound heterozygous BH_4-responsive PAH deficiency

The heteroallelic $Pah^{enu1/2}$ was previously reported as an orthologue for human hyperphenylalaninemia [20] with plasma phenylalanine levels of 147–200 μM and a residual enzyme activity of about 5% as compared to wild-type mice. We revisited this mouse model in order to reevaluate the biochemical phenotype and PAH enzyme function *in vivo* and *in vitro* and to test for responsiveness to BH_4. In our experimental setup mean blood phenylalanine concentrations were 54 ± 2.1 μM for the wild-type, 177 ± 25.7 μM for $Pah^{enu1/1}$, 284 ± 24.9 μM for $Pah^{enu1/2}$, and 1115 ± 54.2 μM for $Pah^{enu2/2}$, in ascending order (Fig. 1A). Phenylalanine oxidation, which is a measure of *in vivo* PAH enzyme activity, was assessed by a ^{13}C-phenylalanine oxidation test [7,8,16]. The cumulative recovery of $^{13}CO_2$ (the product of ^{13}C-phenylalanine oxidation) after 104 min reached 38 ± 2.8% for the wild-type, whereas $Pah^{enu1/1}$ (12 ± 1.0%), $Pah^{enu1/2}$ (9 ± 0.8%), and $Pah^{enu2/2}$ (5 ± 0.6%) showed lower values in descending order (Fig. 1B).

Results obtained from ^{13}C-phenylalanine breath tests were substantiated *in vitro* by overexpression of wild-type, V106A, F263S, and combined overexpression of V106A and F263S PAH, respectively. COS-7 cells transiently transfected with V106A, the *in vitro* model for $Pah^{enu1/1}$, or V106A/F263S, the *in vitro* model for $Pah^{enu1/2}$, showed decreased PAH activity in comparison to the wild-type (Fig. 1C). In line with the *in vivo* experiments, co-transfection of V106A/F263S resulted in a significantly lower enzyme activity than transfection of V106A only. As expected, transfection of F263S resulted in almost no residual enzyme activity.

As a next step, we aimed to determine the response of $Pah^{enu1/2}$ to pharmacological doses of BH_4. A single dose of BH_4 (20 μg/g bw i.p.) significantly reduced blood phenylalanine concentrations of $Pah^{enu1/2}$ from 284 ± 24.9 to 135 ± 17.2 μM and of $Pah^{enu1/1}$ from 177 ± 25.7 to 70 ± 17.2 μM, whereas phenylalanine values remained unchanged in wild-type mice and in $Pah^{enu2/2}$. $Pah^{enu1/1}$ reached blood phenylalanine concentrations close to that of wild-type mice, while $Pah^{enu1/2}$ still displayed mild hyperphenylalaninemia after treatment (Fig. 1D). In addition, BH_4 injection led to an increase in phenylalanine oxidation in $Pah^{enu1/1}$ and $Pah^{enu1/2}$. The cumulative recovery of $^{13}CO_2$ after 104 min attained wild-type level in both $Pah^{enu1/2}$ and $Pah^{enu1/1}$. In agreement with the missing effect on blood phenylalanine concentrations, BH_4 treatment did not affect phenylalanine oxidation rates in $Pah^{enu2/2}$ (Fig. 1E). In wild-type mice, however, BH_4 led to a reduction in the cumulative $^{13}CO_2$ recovery, which is in line with the known inhibitory effect of the cofactor on enzyme activity [34].

In COS-7 cells, treatment with BH_4 over 24 h led to an increase in enzyme activity in cells overexpressing wild-type PAH as well as V106A and V106A/F263S (Fig. 1F) with the effect being most pronounced in cells expressing V106A-PAH.

Taken together, determination of phenylalanine oxidation mirrored the biochemical phenotype and allowed for sensitive discrimination between all genotypes tested. Moreover, combined analysis of the biochemical and molecular phenotype showed that $Pah^{enu1/2}$ displays more severe PAH deficiency than $Pah^{enu1/1}$ and identified $Pah^{enu1/2}$ as a compound heterozygous model for human BH_4-responsive PAH deficiency.

3.2. Pharmacodynamic characterization of BH_4 treatment in wild-type and PAH deficient mice

To characterize the pharmacodynamic effects of BH_4 on wild-type and variant PAH *in vivo* we selected a combined set of three different endpoints: (i) blood phenylalanine elimination, (ii) blood phenylalanine/tyrosine ratios, and (iii) kinetics of *in vivo* phenylalanine oxidation.

In wild-type mice a phenylalanine challenge (15 μg/g bw i.p.) led to no significant changes in blood phenylalanine concentrations (Fig. 2A) or the phenylalanine/tyrosine ratio (Fig. 2B). Accordingly, the ^{13}C-phenylalanine applied was almost completely oxidized to $^{13}CO_2$ within the first hour (Fig. 2C).

In $Pah^{enu1/1}$ and $Pah^{enu1/2}$ the phenylalanine challenge induced a marked increase in both blood phenylalanine concentrations (Fig. 2A) and blood phenylalanine/tyrosine ratios (Fig. 2B) that did not recover to initial values within 3 h (Table 1). In agreement with the respective biochemical (blood phenylalanine concentrations) and functional (phenylalanine oxidation) phenotypes, $Pah^{enu1/2}$ showed more severe alterations of blood phenylalanine elimination and phenylalanine/tyrosine ratio elevations than $Pah^{enu1/1}$.

The effect of BH_4 on phenylalanine elimination and on the phenylalanine/tyrosine ratio was different from that on phenylalanine oxidation, where both $Pah^{enu1/1}$ and $Pah^{enu1/2}$ reached wild-

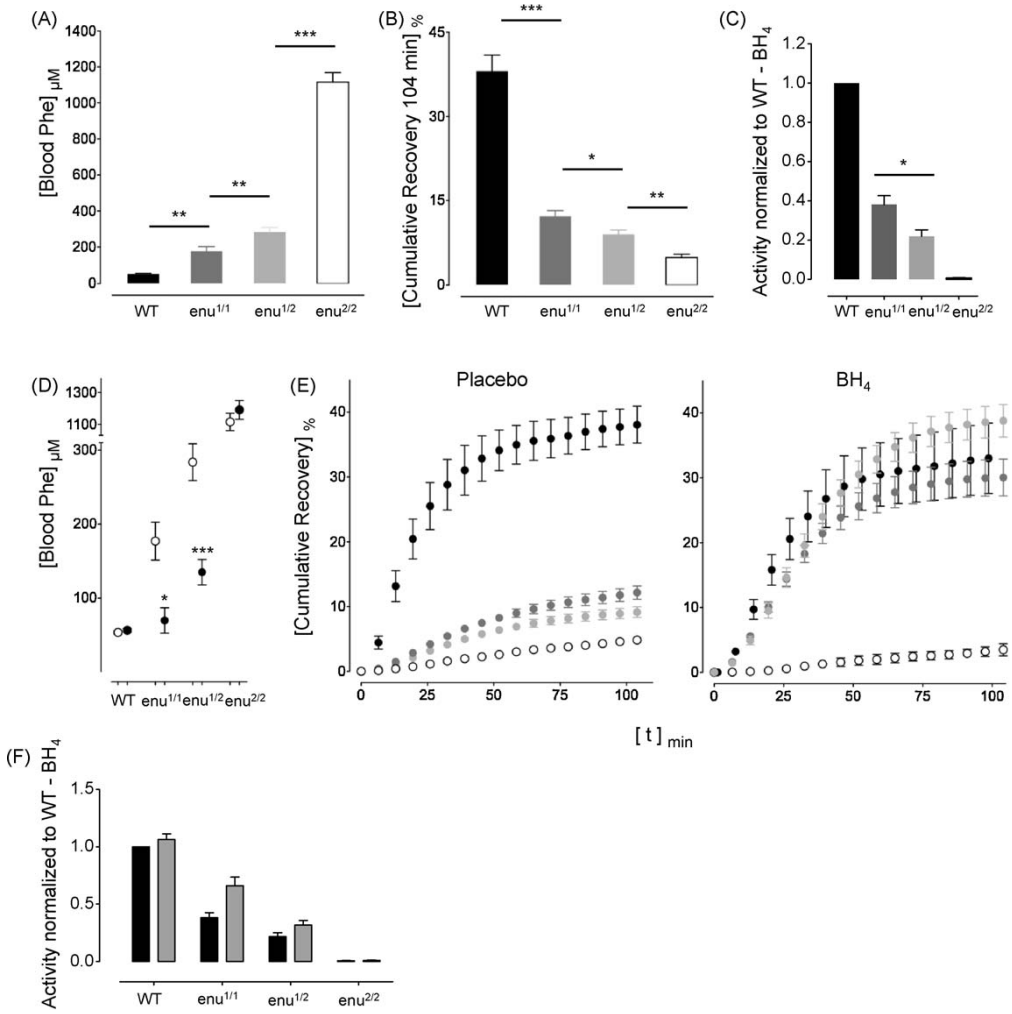

Fig. 1. Characterization of the biochemical and molecular phenotype of wild-type and PAH deficient mice and evaluation of BH_4-responsiveness. (A) Basal levels of blood phenylalanine in the wild-type (WT), *Pah*$^{enu1/1}$ (enu$^{1/1}$), *Pah*$^{enu1/2}$ (enu$^{1/2}$), and *Pah*$^{enu2/2}$ (enu$^{2/2}$). (B) ^{13}C-phenylalanine oxidation *in vivo* assessed in wild-type, *Pah*$^{enu1/1}$, *Pah*$^{enu1/2}$, and *Pah*$^{enu2/2}$ expressed as cumulative $^{13}CO_2$ recovery at 104 min after application of ^{13}C-phenylalanine (15 μg/g bw i.p.). (C) *In vitro* PAH activity in COS-7 cells transiently expressing murine wild-type and variant PAH. Values were normalized to wild-type activity without BH_4 treatment. (D) Blood phenylalanine before (open circles) and 120 min after a single dose of 20 μg/g bw i.p. BH_4 (closed circles). (E) ^{13}C-phenylalanine oxidation *in vivo* assessed in wild-type (black), *Pah*$^{enu1/1}$ (dark grey), *Pah*$^{enu1/2}$ (light grey), and *Pah*$^{enu2/2}$ (white). Data points represent cumulative $^{13}CO_2$ recovery after application of ^{13}C-phenylalanine (15 μg/g bw i.p.) and a simultaneous injection of placebo (sodium chloride 0.9%, ascorbic acid 1%) or BH_4 (20 μg/g bw i.p.). Samples were collected in 6.5-min intervals over a 104-min period. (F) *In vitro* PAH activity in COS-7 cells transiently expressing murine wild-type and variant PAH upon 24 h incubation with BH_4 (43 μM, grey bars) and without BH_4 supplementation (black bars). Values were normalized to wild-type activity without BH_4 supplementation. All data are given as means ± s.e.m. and the significance is indicated (*$P < 0.05$, **$P < 0.01$, ***$P < 0.001$).

type levels after treatment (Fig. 1E). Only *Pah*$^{enu1/1}$ showed normalization of phenylalanine elimination and the phenylalanine/tyrosine ratio within 120 and 60 min, respectively. Both parameters did not significantly decrease in *Pah*$^{enu1/2}$ and the blood phenylalanine concentration and the phenylalanine/tyrosine ratio remained elevated 3 h after BH_4 administration (Table 1).

In both animal models the rates of phenylalanine oxidation, assessed by determination of delta over baseline, exceeded half maximum values as early as 6.5 min post-BH_4 injection. However, *Pah*$^{enu1/1}$ reached maximum oxidation rates at 13 min, whereas *Pah*$^{enu1/2}$ displayed a delayed maximum at 26 min (Fig. 2C). The resulting elimination constants (K_e) for blood phenylalanine again showed lower response to the drug for *Pah*$^{enu1/2}$ (0.014) than for *Pah*$^{enu1/1}$ (0.023) (Table 1).

In summary, the application of the three endpoints presented here allowed for a clear discrimination in pharmacodynamics between the two mouse strains. Differences in the response to the drug were not only confined to the effect size but also observed in terms of effect kinetics.

3.3. Pharmacokinetics of BH_4

In order to determine, whether differences in pharmacodynamics were induced by discrepant pharmacokinetic behavior, we quantified total biopterin blood concentrations over time after intraperitoneal injection of 20 μg/g bw of BH_4. We observed an increase from 19.4 ± 3.1 to 467.4 ± 47.5 nmol/g Hb in *Pah*$^{enu1/1}$ and from 17.3 ± 2.5 to 558.8 ± 68.3 nmol/g Hb in *Pah*$^{enu1/2}$. The maximum was reached after 17.5 min and concentrations decreased rapidly thereafter. About 70% of exogenous BH_4 was eliminated 60 min post-application followed by a second slow elimination phase that reached initial values (*Pah*$^{enu1/1}$ 51.2 ± 6.2, *Pah*$^{enu1/2}$ 55.6 ± 6.7) at 180 min. None of the pharmacokinetic parameters (c_0, c_{max}, t_{max}, AUC, $t_{1/2}$) significantly differed between *Pah*$^{enu1/1}$ and *Pah*$^{enu1/2}$ (Table 2).

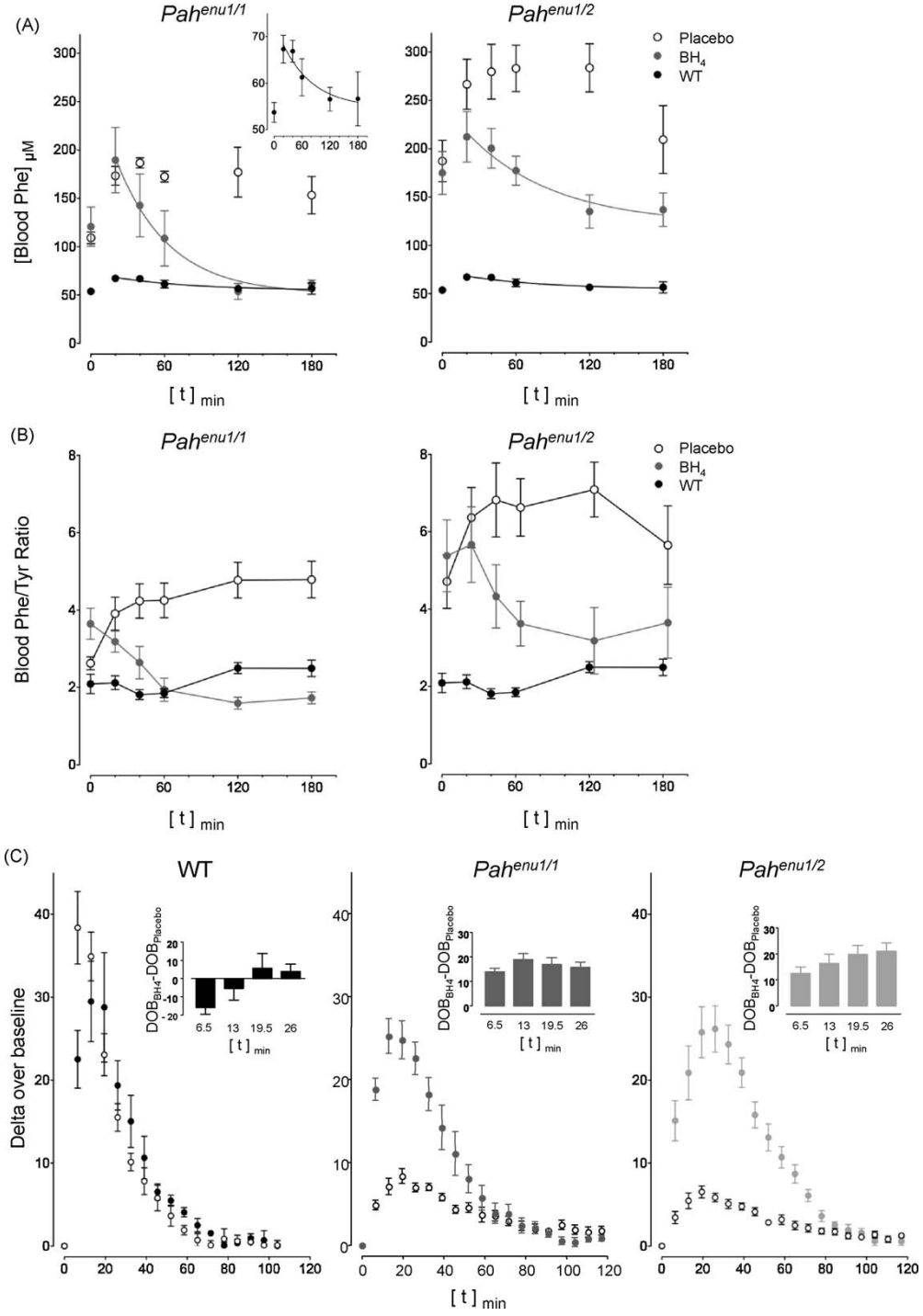

Fig. 2. Characterization of BH_4 effects on PAH function *in vivo*. (A) Blood phenylalanine elimination and (B) blood phenylalanine/tyrosine ratio (Phe/Tyr ratio) were assessed in $Pah^{enu1/1}$ and $Pah^{enu1/2}$ mice in comparison to untreated wild-type mice. The effect of simultaneous challenge of unlabelled phenylalanine (15 μg/g bw) and BH_4 (20 μg/g bw) vs. placebo (sodium chloride 0.9%, ascorbic acid 1%) was measured over a 180-min period and data points representing the decrease in blood phenylalanine concentrations were fitted using a one-phase exponential function. The inset represents blood phenylalanine elimination in wild-type mice at a different scale. (C) Fractional $^{13}CO_2$ recovery in ^{13}C-phenylalanine oxidation tests (delta over baseline, DOB) of wild-type mice (WT), $Pah^{enu1/1}$ and $Pah^{enu1/2}$. The measurement was performed in 6.5-min intervals over a 104-min period after simultaneous injection of BH_4 (20 μg/g bw) and phenylalanine (15 μg/g bw) (closed circles) or placebo (open circles). To depict the immediate onset of the BH_4 effects, the differences of fractional recovery (DOB_{BH4}–$DOB_{placebo}$) within the first four time points are given in the insets. All data are given as means ± s.e.m.

Table 1
Phenylalanine kinetics and phenylalanine/tyrosine ratios.

	BH_4	Phe_0 [μmol/l]	Phe_{180} [μmol/l]	*P* value	K_e	Phe/Tyr_0	Phe/Tyr_{180}	*P* value
Pah$^{enu1/1}$	–	109.10 ± 5.4	153.33 ± 19.3	0.0097	–	2.62 ± 0.1	4.79 ± 0.4	0.0035
	+	120.74 ± 16.5	61.64 ± 3.9		0.023	3.65 ± 0.3	1.73 ± 0.1	
Pah$^{enu1/2}$	–	187.29 ± 19.8	209.50 ± 35.1	n.s.	–	4.71 ± 0.6	5.65 ± 1.0	n.s.
	+	174.94 ± 20.6	136.9 ± 17.3		0.014	5.38 ± 0.8	3.65 ± 0.9	
BTBR	–	53.70 ± 1.8	56.61 ± 5.0	–	0.015	2.15 ± 0.2	2.49 ± 0.2	-

Phenylalanine kinetics and phenylalanine/tyrosine ratios were determined after a phenylalanine load of 15 μg/g bw i.p. with and without simultaneous BH_4 load (20 μg/g bw i.p.) to wild-type (BTBR), *Pah*$^{enu1/1}$ and *Pah*$^{enu1/2}$. Phenylalanine concentrations and phenylalanine/tyrosine ratios are given as means ± s.e.m. The elimination constant (K_e) was determined using a one-phase exponential function. Unpaired two-tailed students *t*-tests were applied to test the difference between untreated and treated animals (n.s., not significant).

3.4. Effect duration of BH_4

We subsequently aimed to analyze, whether BH_4 effect duration diverges between *Pah*$^{enu1/1}$ and *Pah*$^{enu1/2}$. For this purpose we monitored the time course of ^{13}C-phenylalanine oxidation after a single dose of BH_4 and analyzed the effect of BH_4 on PAH activity in cultured cells over 72 h.

In both PAH deficient strains phenylalanine oxidation reached its maximum immediately after BH_4 application (Fig. 2C). In order to compare effect durations we normalized the data defining the highest values as 100% and the placebo level as 0%. A single dose of 40 μg/g bw BH_4 induced enhanced phenylalanine oxidation for more than 48 h in *Pah*$^{enu1/1}$, while in *Pah*$^{enu1/2}$ phenylalanine oxidation decayed to placebo levels within 18 h (Fig. 3A).

These results were confirmed by assessing the long-term effect of BH_4 on PAH enzyme activity in COS-7 cells (Fig. 3B). In cells expressing V106A, BH_4 treatment normalized enzyme activity, while cells expressing V106A/F263S reached 75% of wild-type activity. BH_4 did not affect enzyme activity in cells transfected with F263S. In addition, we compared the fold increase of enzyme activity over the control (no BH_4 treatment) after 24 and 72 h of cell incubation with BH_4. At overexpression of V106A the major part of the response occurred within 24 h (1.54-fold increase) and cultivation for additional 48 h did not substantially further increase enzyme activity (1.75-fold). By contrast, cells expressing V106A/F263S showed their main increase in enzyme activity (1.9-fold) after prolonged cultivation of 72 h and not after 24 h (1.15-fold) (Fig. 3C).

In conclusion, *Pah*$^{enu1/1}$ and *Pah*$^{enu1/2}$ showed pronounced differences in effect duration of BH_4 treatment. The *in vivo* effect of BH_4 was shorter in *Pah*$^{enu1/2}$ than in *Pah*$^{enu1/1}$. This was demonstrated by showing that the functional effect of the drug on phenylalanine oxidation leveled off more rapidly. *In vitro*, a prolonged treatment was needed to reach the maximum treatment effect in *Pah*$^{enu1/2}$.

3.5. Dose effects of BH_4

To further characterize genotype-specific pharmacodynamics we studied the dose response to BH_4. Analysis of the cumulative recovery of ^{13}C-phenylalanine oxidation at 104 min upon single dose BH_4 treatment revealed a non-dose-dependent inhibitory effect in the range of 5–40 μg/g bw i.p in wild-type mice (Fig. 4A). *Pah*$^{enu1/1}$ showed a positive dose response starting at 5 μg/g and reached a plateau at 20 μg/g with no further increase in phenylalanine oxidation at higher doses (Fig. 4B). In the compound heterozygous mouse *Pah*$^{enu1/2}$ we also identified a positive dose effect. However, BH_4 doses of 30 and 40 μg/g resulted in a lower activity as compared to the peak effect at 10–20 μg/g (Fig. 4C). With respect to the effect size *Pah*$^{enu1/2}$ revealed a stronger response as compared to *Pah*$^{enu1/1}$ at 5, 10, and 20 μg/g, respectively (Fig. 4B and C).

In conclusion, *Pah*$^{enu1/1}$ and *Pah*$^{enu1/2}$ showed a dose dependent and increasing response to BH_4 at low doses up to 10 μg/g with no further benefit at higher dosages. Moreover, the results observed for *Pah*$^{enu1/2}$ are in line with a negative dose effect for concentrations above 20 μg/g BH_4.

Table 2
Determination of pharmacokinetic parameters.

	Pah$^{enu1/1}$	*Pah*$^{enu1/2}$	*P* value
c_0 (nmol/g Hb)	19.4 ± 3.1	17.3 ± 2.5	0.5
c_{max} (nmol/g Hb)	467.6 ± 47.5	558.8 ± 68.3	0.3
t_{max} (min)	17.5 ± 2.5	17.5 ± 2.5	1
AUC_{180min}	23769 ± 2341	24522 ± 2638	0.8
$t_{1/2}$ (min)	26.3 ± 2.8	26.3 ± 2.7	0.98

The following pharmacokinetic parameters were determined in *Pah*$^{enu1/1}$ and *Pah*$^{enu1/2}$: c_0 initial concentration, c_{max} peak concentration, t_{max} time to peak concentration, AUC area under the concentration vs. time curve at 0–180 min, $t_{1/2}$ elimination half-life.

4. Discussion

The approval of BH_4 marked a paradigm change in the management of phenylketonuria treatment. Clinical studies performed to analyze BH_4-responsiveness in patients often focused on the surrogate marker blood phenylalanine concentration as an endpoint. However, this method produced high intraindividual variability and discrepant findings among similar genotypes [23] and therefore proved to be of limited value. Moreover, an animal model with the specific clinical and biochemical phenotype of BH_4-responsive PAH deficiency was not available during the development of the drug. Thus, important insights into pharmacodynamics of BH_4 were still lacking, in particular with regard to pharmacogenetics as well as effect size and effect kinetics.

We recently characterized *Pah*$^{enu1/1}$ (V106A/V106A) as the first animal model for BH_4-responsive MHP [16]. This opened up the opportunity for first *in vivo* investigations in order to elucidate the BH_4 mode of action and to substantiate the view of the cofactor being a pharmacological chaperone. The genetic alteration in this mouse does not lead to changes in PAH affinity to the cofactor [16] reflecting the human situation where only few K_m variants were found [4,14,15,35–37]. Thus, the mode of action of BH_4 in PAH deficiency was not limited to its cofactor action. We showed that pharmacological doses of BH_4 attenuate the pathophysiological triad of misfolding, aggregation, and accelerated degradation of the PAH enzyme by conformational stabilization augmenting the effective PAH concentration. This led to the rescue of the biochemical phenotype and enzyme function *in vivo*. Notably, the pharmaceutical action of the cofactor was confined to the pathological metabolic state of hyperphenylalaninemia [16].

Here we present *Pah*$^{enu1/2}$ (V106A/F263S) as a second mouse model for BH_4-responsive PKU. This strain displays compound heterozygosity representing the genotype of the large majority of PKU patients (87%) [23] that are dealt with in daily clinical routine.

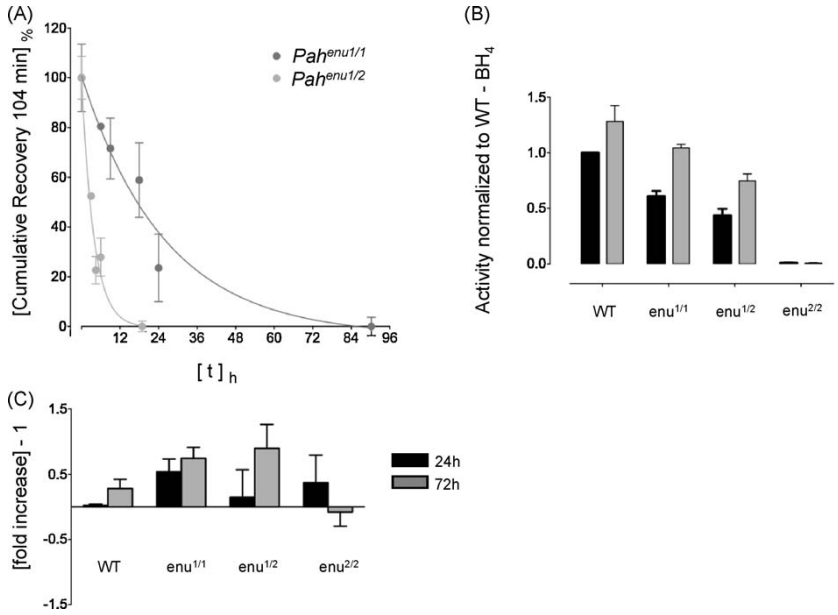

Fig. 3. *In vivo* and *in vitro* characterization of BH_4 effect duration. (A) Effect duration of BH_4 in $Pah^{enu1/1}$ and $Pah^{enu1/2}$ mice expressed as relative recovery of $^{13}CO_2$ at 104 min. BH_4 was administered 90, 24, 18, 9, 6, 4.5 or 3 h before or simultaneously to ^{13}C-phenylalanine and recovery of $^{13}CO_2$ was measured subsequently. Data were normalized defining the maximum recovery as 100% and the placebo level as 0%. Lines depict the difference in phenylalanine oxidation and are merely to guide the eye. (B) *In vitro* PAH activity in COS-7 cells transiently expressing murine wild-type and variant PAH upon 72 h incubation with BH_4 (43 μM, grey bars) and without BH_4 supplementation (black bars). Values were normalized to wild-type activity without BH_4 supplementation. (C) Comparison of *in vitro* PAH enzyme activities. Fold increase describes the ratio between enzyme activities determined with and without addition of BH_4 (43 μM) after 24 h (black bars) and 72 h (grey bars) incubation. All data are given as means ± s.e.m.

Amino acid residues V106 in the regulatory domain and F263 in the catalytic core are conserved between mouse and human but neither V106A nor F263S have so far been identified in human PKU patients. However, the F263L amino acid substitution was described to be also associated with a severe phenotype. A comparison of specific activities of murine and human PAH variants V106A, F263S, and F263L confirmed that amino acid substitutions at the respective loci result in similar effects in both species (Supplementary Table S1). Thus, murine V106A and F263S variants are good models for human BH_4-responsive PAH deficiency.

As previously shown [20] and confirmed in our current study, $Pah^{enu1/2}$ exhibits an intermediate phenotype between $Pah^{enu1/1}$ and $Pah^{enu2/2}$. Hyperphenylalaninemia *in vivo* is more severe than in $Pah^{enu1/1}$ and less severe than in $Pah^{enu2/2}$, whereas PAH activity *in vivo* and *in vitro* is lower than in $Pah^{enu1/1}$ but higher than in $Pah^{enu2/2}$. Analyses of phenylalanine oxidation by ^{13}C-phenylalanine breath tests mirrored these differences in severity and were in good agreement with biochemical (blood phenylalanine) and molecular (PAH activity) data allowing for high-sensitivity discrimination of genotypes.

We compared pharmacodynamics of BH_4 treatment in the two mouse models and observed crucial differences. Heterogeneous results were obtained with respect to the effect size. In the combined phenylalanine-BH_4-loading test a single load of 20 μg/g bw BH_4 induced a decrease of blood phenylalanine of 67.5% in $Pah^{enu1/1}$ and of only 35.5% in $Pah^{enu1/2}$. This value is in close proximity of the threshold of 30%, an arbitrary but accepted measure for BH_4-responsiveness in humans considered to be associated with clinical benefit. By contrast, the effect on *in vivo* phenylalanine oxidation, the direct measure of PAH activity, was striking in this strain. Treatment with BH_4 completely normalized *in vivo* enzyme activity with the effect being even stronger than in the milder phenotype $Pah^{enu1/1}$.

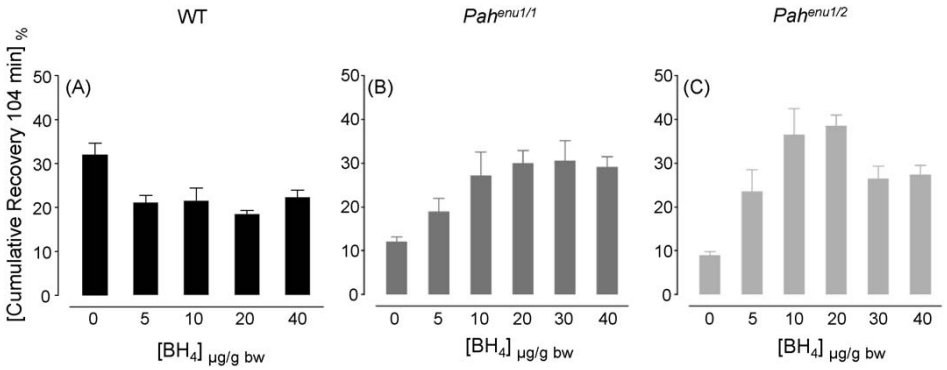

Fig. 4. Dose effect of BH_4 *in vivo*. Effect of BH_4 on phenylalanine oxidation expressed as cumulative recovery at 104 min measured in wild-type (WT) (A), $Pah^{enu1/1}$ (B), and $Pah^{enu1/2}$ (C). Data are given as means ± s.e.m.

Analysis of the effect kinetics further added to dissect the distinct patterns of BH_4-response in the two strains. At the biochemical level, *Pah*$^{enu1/2}$ showed slower elimination of pathologically elevated phenylalanine concentrations than *Pah*$^{enu1/1}$. This was mirrored by a less pronounced and delayed reduction of the phenylalanine/tyrosine ratio, an important parameter to judge the extent of metabolic derangement. At the functional level, both strains showed an immediate response to BH_4-loading in the breath test, but the peak phenylalanine oxidation upon treatment again was delayed in *Pah*$^{enu1/2}$ in comparison to *Pah*$^{enu1/1}$. Moreover, the duration of the BH_4 effect after a single dose was considerably shorter in *Pah*$^{enu1/2}$ than in *Pah*$^{enu1/1}$.

In addition, the two animal models displayed important divergences concerning dose response. Both strains attained maximum *in vivo* phenylalanine oxidation at a dose of 20 μg/g bw BH_4. Yet, *Pah*$^{enu1/2}$ exhibited a response inhibition in the presence of higher dosages, while *Pah*$^{enu1/1}$ reached a plateau. This data is in line with well known features of pharmacological chaperones which show inhibitory effects at higher concentrations [38].

Our findings may lead to important conclusions concerning the diagnostic and therapeutic management of patients with PAH deficiency. First, extending the test procedures to assess BH_4-responsiveness may allow to draw a more complete picture of the drug response in the single individual. The methods presented in this work can easily and safely be transferred into clinical routine and provide useful endpoints beyond determination of blood phenylalanine concentrations. Second, some patients show a delayed reduction in blood phenylalanine after BH_4-loading, these are often referred to as slow responders [8,22,25,39]. Our results indicate that this may be rather due to a limited pharmacodynamic effect than to a delayed onset of drug action. Cell culture experiments pointed to a beneficial effect of prolonged treatment for slow responder genotypes and hence suggest appreciation of this fact when testing for and treating BH_4-responsive PAH deficiency. Third, both animal models bear the same mutation responsible for BH_4-responsiveness (V106A), however, the null-mutation on the second allele in the compound heterozygous genotype *Pah*$^{enu1/2}$ had substantial impact on pharmacodynamics. Thus, response to treatment is not exclusively related to the putative milder mutation [23,24,39] and effects induced by interallelic complementation may necessitate careful dose finding procedures in compound heterozygous patients. Fourth, the individual behavior with regard to effect size, onset of maximum drug action, and effect duration underscores the demand of individual therapeutic regimes for different genotypes. Some patients may for instance benefit from a treatment scheme with several BH_4 administrations to translate the effect of the drug on enzyme activity into a sustained effect on biochemical markers. Moreover, in certain patients, higher dosages may diminish the positive treatment effect at a higher risk of possible adverse effects.

In conclusion, considerable clinical and research effort has been devoted to identifying the conditions for optimal testing for BH_4-responsiveness in PAH deficiency. Our in depth pharmacological analyses of two mouse models with different genotypes are in line with the notion that genotype-driven complexity will require comprehensive evaluation instruments addressing different pharmacodynamic and pharmacokinetic aspects. For this purpose, the diagnostic package including time-dependent blood phenylalanine elimination and phenylalanine/tyrosine ratios as well as kinetics of *in vivo* phenylalanine oxidation can easily be transferred to and implemented in patients and by this may contribute to individualized diagnostics and treatment of patients suffering from a genetically heterogeneous condition.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bcp.2010.07.042.

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Table S1. Steady state enzyme kinetic parameters of wild-type and variant murine PAH proteins

Genotype	Specific activity (nmol Tyr/min x mg protein)
WT hsPAH	3115 ± 149
WT MmPAH	2628 ± 293
V106A HsPAH	2947 ± 260
V106A MmPAH	3125 ± 101
F263S HsPAH	235 ± 51
F263L HsPAH	159 ± 51
F263S MmPAH	166 ± 16

Recombinant tetrameric human (HsPAH) and murine (MmPAH) PAH were expressed as MBP-PAH fusion proteins in *E. coli*. Specific activity was determined at standard L-phenylalanine (1 mM) and BH_4 (75 µM) concentrations with L-phenylalanine preincubation. Values are given as means ± SEM of *n* = 3 experiments.

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ORIGINAL ARTICLE

Secondary BH_4 deficiency links protein homeostasis to regulation of phenylalanine metabolism

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Abstract

Metabolic control of phenylalanine concentrations in body fluids is essential for cognitive development and executive function. The hepatic phenylalanine hydroxylating system is regulated by the ratio of L-phenylalanine, which is substrate of phenylalanine hydroxylase (PAH), to the PAH cofactor tetrahydrobiopterin (BH_4). Physiologically, phenylalanine availability is governed by nutrient intake, whereas liver BH_4 is kept at constant level. In phenylketonuria, PAH deficiency leads to elevated blood phenylalanine and is often caused by PAH protein misfolding with loss of function. Here, we report secondary hepatic BH_4 deficiency in Pah-deficient mice. Alterations in *de novo* synthesis and turnover of BH_4 were ruled out as molecular causes. We demonstrate that kinetically instable and aggregation-prone variant Pah proteins trap BH_4, shifting the pool of free BH_4 towards bound BH_4. Interference of PAH protein misfolding with metabolite-based control of L-phenylalanine turnover suggests a mechanistic link between perturbation of protein homeostasis and disturbed regulation of metabolic pathways.

Introduction

The pteridine derivative tetrahydrobiopterin (BH_4; 6*R*-L-*erythro*-5,6,7,8-tetrahydrobiopterin) is an essential cofactor for hydroxylation of L-phenylalanine (L-Phe) to L-tyrosine by the enzyme phenylalanine hydroxylase (PAH; EC 1.14.16.1). Hepatocytes are the main site of metabolic clearance of circulating L-Phe. In addition, RNA transcripts have been found in the kidney, the pancreas and the brain (1,2); however, the relevance of the phenylalanine hydroxylating system in these tissues remains unclear. Deficiency of the PAH enzyme due to sequence variation in the *PAH* gene (NCBI Gene ID 5053) causes the biochemical phenotype of hyperphenylalaninemia, i.e. elevated L-Phe concentrations in the blood and body fluids. The resulting disease phenylketonuria (PKU; OMIM 261600) is the most common inherited disorder of amino acid metabolism with a prevalence of 1–5 in 10 000 individuals in the European descendent population (3–6). Residual PAH activity *in vivo* determines the clinical phenotype of PKU patients, ranging from severe classical PKU via mild PKU to mild hyperphenylalaninemia, with increasing activity and decreasing blood L-Phe concentrations (7–9).

Binding of BH_4 and L-Phe to PAH is complexly regulated (10–12). While the blood L-Phe concentration, even in healthy individuals, is subject to changes of up to 50% within 24 h, depending on nutritional intake and the metabolic state (13), the concentration of BH_4 is constantly kept at a level of 8–12 μM in hepatocytes (14,15). High concentrations of L-Phe lead to substrate-activation of the PAH enzyme, which is induced by allosteric conformational rearrangements (16–20). In

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contrast, binding of BH_4 to non-activated PAH induces a resting t-state (21–23), hence, BH_4 inhibits PAH activity at low L-Phe concentrations. Therefore, binding of BH_4 to PAH depends on the availability of L-Phe and the conformational state of the PAH enzyme. As a consequence, two pools of hepatic BH_4 exist, free BH_4 and BH_4 bound to PAH (15). Moreover, residual enzyme activity in PKU patients is driven by the interplay of L-Phe and BH_4 concentrations (24).

Loss of PAH enzymatic function has been shown to result from misfolding, conformational destabilization and rapid degradation, as a consequence of amino acid side-chain replacements in the PAH protein (25–27). The discovery of PAH conformational stabilization by BH_4-binding enabled implementation of a pharmacological treatment for PKU that addresses the loss-of-function molecular phenotype (28–30). The active compound sapropterin dihydrochloride, a synthetic form of BH_4, is not efficacious for all PKU patients (28,31) and drug response depends on the underlying genotype (32–35). In the group of patients treated with pharmacological doses of BH_4, not only L-Phe concentrations but also blood BH_4 concentrations undergo significant changes (36–38). Pharmacokinetics of BH_4 following oral administration have been studied in healthy subjects (36) as well as in PKU patients (37–39) and high intra-individual and inter-individual variability of blood BH_4 was observed. Animal studies showed accumulation of BH_4 in liver and kidney after oral or intravenous application (40–42). However, the impact of the genotype and of therapeutic BH_4 supplementation on biopterin levels in the liver, where the phenylalanine hydroxylating system is mainly active, is not well understood and pools of liver BH_4 have not been investigated *in vivo*.

Two PKU mouse models carrying nucleotide substitutions in the murine *Pah* gene are available (43). The loss-of-function variant c.835T>C (p.Phe263Ser), which affects the catalytic core of the murine Pah enzyme, leads to a severe classical PKU phenotype in $Pah^{enu2/2}$ mice. In contrast, the mild hyperphenylalaninemia phenotype observed for $Pah^{enu1/1}$ is due to the variant c.364T>C (p.Val106Ala), which induces misfolding and degradation of murine Pah (44). The hybrid $Pah^{enu1/2}$ was described as a model for compound heterozygous PAH deficiency with a mild PKU phenotype (45,46).

We analyzed the phenylalanine hydroxylating system of all three murine *Pah* genotypes and observed decreased levels of BH_4 in mice carrying the Pah^{enu1} allele (p.Val106Ala) but not in wild-type or animals homozygous for the severe variant c.835T>C (p.Phe263Ser). *In vitro* experiments using purified variant murine Pah demonstrated trapping of BH_4 by the misfolded Pah variant p.Val106Ala. Secondary BH_4 deficiency due to disturbed BH_4 homeostasis is a new molecular mechanism contributing to the complex regulatory network of phenylalanine metabolism.

Results

Secondary BH_4 deficiency in $Pah^{enu1/1}$ and $Pah^{enu1/2}$

To investigate the liver phenylalanine hydroxylating system independent of blood-derived factors, we isolated hepatocytes and determined Pah activity and BH_4 concentrations *ex vivo*. Pah activities were compared with concentrations of L-Phe and BH_4 in the blood of donor animals. Genotype-specific hepatic Pah activity observed in isolated hepatocytes (Fig. 1A, Supplemetary Material, Table S1) was in line with Pah enzyme activity measured in whole liver lysates (44,47). $Pah^{enu1/1}$ and $Pah^{enu1/2}$ exhibited 11.5 and 7% residual activity, respectively, as compared to the wild-type. Almost no residual activity was determined in $Pah^{enu2/2}$ animals (0.7%). Residual activity of murine genotypes inversely correlated with blood L-Phe levels (Fig. 1B, Supplementary Material, Table S1). $Pah^{enu2/2}$ with almost no detectable Pah activity showed blood L-Phe concentrations above 1200 μmol/l that correspond to the classic PKU phenotype. $Pah^{enu1/1}$ and $Pah^{enu1/2}$, associated with low Pah activity, exhibited L-Phe levels in the range of mild hyperphenylalaninemia. Elevated levels of blood L-Phe induce upregulation of GTP cyclohydrolase 1, the rate-limiting enzyme in BH_4 *de novo* synthesis (48,49). The pattern of blood L-Phe concentrations was comparable to the pattern of blood BH_4 levels determined in different murine PKU genotypes (Fig. 1C, Supplementary Material, Table S1). Significantly elevated blood BH_4 concentrations were observed for $Pah^{enu2/2}$ (0.6 μmol/l) compared with wild-type $Pah^{BTBR/BTBR}$ (0.21 μmol/l) (Fig. 1C, Supplemetary Material, Table S1), while there was only a slight increase for $Pah^{enu1/1}$ (0.29 μmol/l) and $Pah^{enu1/2}$ (0.32 μmol/l). In contrast to similarities observed for blood L-Phe and blood BH_4, we found a differing pattern for hepatic BH_4 concentrations (Fig. 1D, Supplementary Material, Table S1). In comparison to wild-type mice (2 ± 1 nmol/g), a decrease of hepatic BH_4 was determined for $Pah^{enu1/1}$ and $Pah^{enu1/2}$ (0.7 and 0.5 nmol/g, respectively) but not for $Pah^{enu2/2}$ (2.9 nmol/g).

To rule out alterations in liver BH_4 metabolism for murine genotypes harboring the Pah^{enu1} allele, we analyzed *Gch1* mRNA, coding for GTP cyclohydrolase 1 (49), in isolated hepatocytes (Fig. 1E, Supplementary Material, Table S1). We found a significant increase in *Gch1* mRNA for $Pah^{enu2/2}$ by 2-fold, which correlated with the increase in blood BH_4. The two genotypes exhibiting mild hyperphenylalaninemia, $Pah^{enu1/1}$ and $Pah^{enu1/2}$, did not show significant alteration of *Gch1* expression. Consequently, decreased concentrations of hepatic BH_4 in $Pah^{enu1/1}$ and $Pah^{enu1/2}$ were not due to down-regulation of GTPCH synthesis, hence, the presence of the Pah^{enu1} allele may modulate hepatic BH_4 concentration through a different mechanism.

Variant-specific BH_4 requirement and coupling of the catalytic reaction

Next, we investigated whether low hepatic BH_4 in $Pah^{enu1/1}$ is due to a different need for BH_4 in the enzymatic reaction. The function of purified recombinant Pah proteins was assessed at a range of different metabolic and therapeutic settings by simultaneous variation of substrate and cofactor concentrations. Enzyme activity was color-coded and plotted as a function of varying L-Phe and BH_4 concentrations (Pah activity landscapes) (Fig. 2A). Pah activity landscapes allow for analysis of peak enzyme activities and optimal working ranges (Table 1), as well as for evaluation of kinetic parameters (Table 2) (24,34). Peak enzyme activity of the purified variant p.Val106Ala, encoded by the Pah^{enu1} allele, reached 75% of the wild-type level (Fig. 2A, Table 1). This was in line with the observation that specific enzyme activity of purified p.Val106Ala is conserved, whereas residual activity *in vivo* of $Pah^{enu1/1}$ is low (44). Activity landscapes revealed a difference in the position of the peak enzyme activity with respect to L-Phe (p.Val106Ala, 561 μM; wild-type, 892 μM), i.e. optimal Pah function is reached at lower L-Phe concentrations for p.Val106Ala as compared to the wild-type. However, we found no evidence for a different BH_4 requirement at peak activity (p.Val106Ala, 137 μM; wild-type, 141 μM). Moreover, the optimal BH_4-dependent working range [(31) $(C)_{0.5} - K_i$; Table 1] and the apparent affinity to BH_4 (l; Table 2) were comparable for p.Val106Ala and the wild-type protein. For the variant p.Phe263Ser, encoded by the Pah^{enu2} allele, no enzyme activity

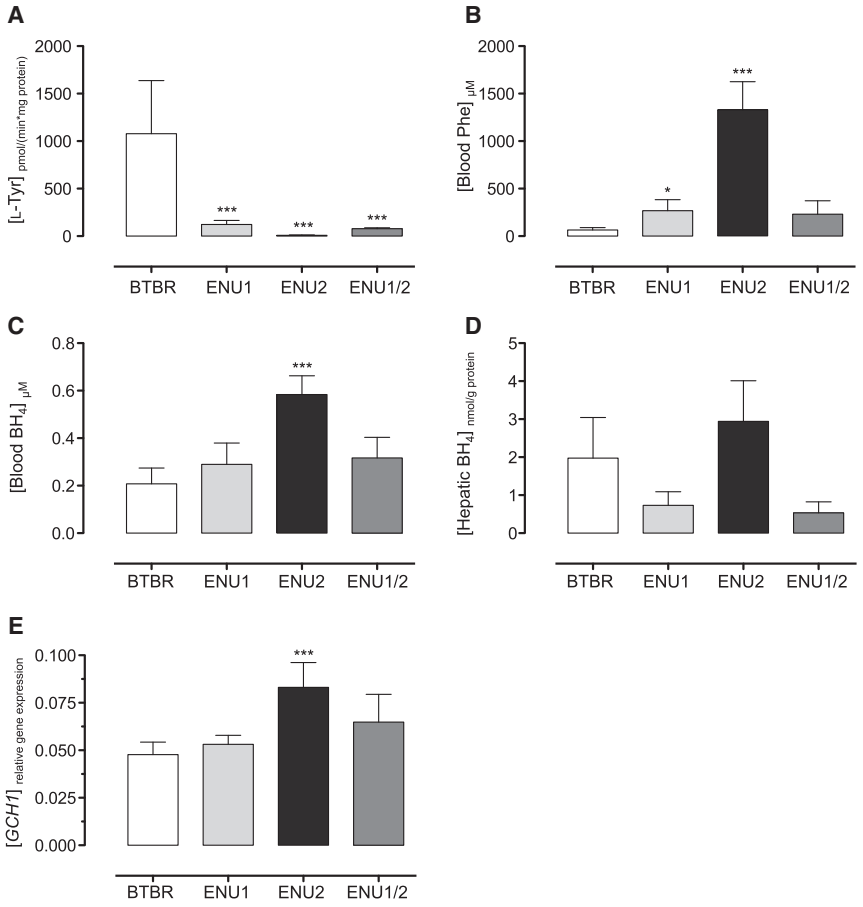

Figure 1. Phenotyping and biochemical characterization of Pah deficient mouse models. *BTBR* (wild-type), *Pah*$^{enu1/1}$ (ENU1; mild PKU phenotype), *Pah*$^{enu2/2}$ (ENU2; severe PKU phenotype) and *Pah*$^{enu1/2}$ (ENU1/2; intermediate phenotype). (**A**) Pah enzyme activity determined in primary hepatocytes extracted from murine livers (n, 5) (**B**) Blood phenylalanine (L-Phe) concentrations of *BTBR* (n, 10), *ENU1* (n, 8), *ENU2* (n, 8) and *ENU1/2* (n, 9) (**C**) Blood tetrahydrobiopterin (BH_4) concentrations in Pah deficient mouse strains (n, 3) (**D**) Tetrahydrobiopterin correlated to total hepatic protein amount in primary hepatocytes extracted from murine livers (n, 3 for each mouse strain) (**E**) Murine *Gch1* gene expression in primary murine hepatocytes. Levels of gene expression were normalized to the hepatic housekeeper gene *Gapdh* ($n = 3$). Significant differences compared to the wild-type (WT) are indicated (***$P \leq 0.001$, **$P < 0.01$, *$P \leq 0.05$, ns $P > 0.05$) for all experiments. Values are given as means ± SD.

was detected, which is in line with *ex vivo* data from isolated hepatocytes.

Under specific conditions, the Pah enzyme catalyzes BH_4 cofactor oxidation without concomitant L-Phe hydroxylation (50). This 'uncoupled' reaction leads to a non-stoichiometric consumption of BH_4, which has been reported for variant human PAH proteins (51). To assess potential differences in BH_4 consumption and BH_2 production between murine wild-type and p.Val106Ala Pah, we performed activity assays with subsequent analyses of BH_4 and BH_2 concentrations in the assay samples (Fig. 2B–D, Supplementary Material, Table S2). Pah activities determined at standard conditions (1 mM L-Phe; 75 µM BH_4) were comparable to data from activity landscapes. Wild-type Pah and p.Val106Ala showed lower BH_4 concentrations in assay samples as compared to the no-protein control. This is in line with BH_4 consumption due to Pah enzyme activity. The oxidation rates of BH_4 to BH_2 correlated with the rates of L-Phe hydroxylation. For the enzymatically inactive variant p.Phe263Ser, the oxidation rate of BH_4 to BH_2 was at the level of the control.

When viewed in combination, these functional studies did not give evidence for a different need for BH_4 or uncoupling of the catalytic reaction as molecular mechanisms for secondary BH_4 deficiency in *Pah*$^{enu1/1}$.

Missense variant-induced misfolding and aggregation of p.Val106Ala controls intracellular BH_4 availability

With the aim to investigate the cause of BH_4 deficiency independent of factors present in isolated hepatocytes, we expressed murine wild-type Pah, p.Val106Ala or p.Phe263Ser in the eukaryotic fibroblast cell line COS-7, which lacks BH_4 *de novo* synthesis. COS-7 cells convert the precursor sepiapterin to BH_4 via the pterin salvage pathway (52,53). When cells were incubated with 40 µM of sepiapterin for 24 h, a variant-specific pattern of intracellular accumulation of free BH_4 was observed (Fig. 3A, Supplementary Material, Table S3). Not transfected controls showed a BH_4 concentration (44 µM) at the level of the applied

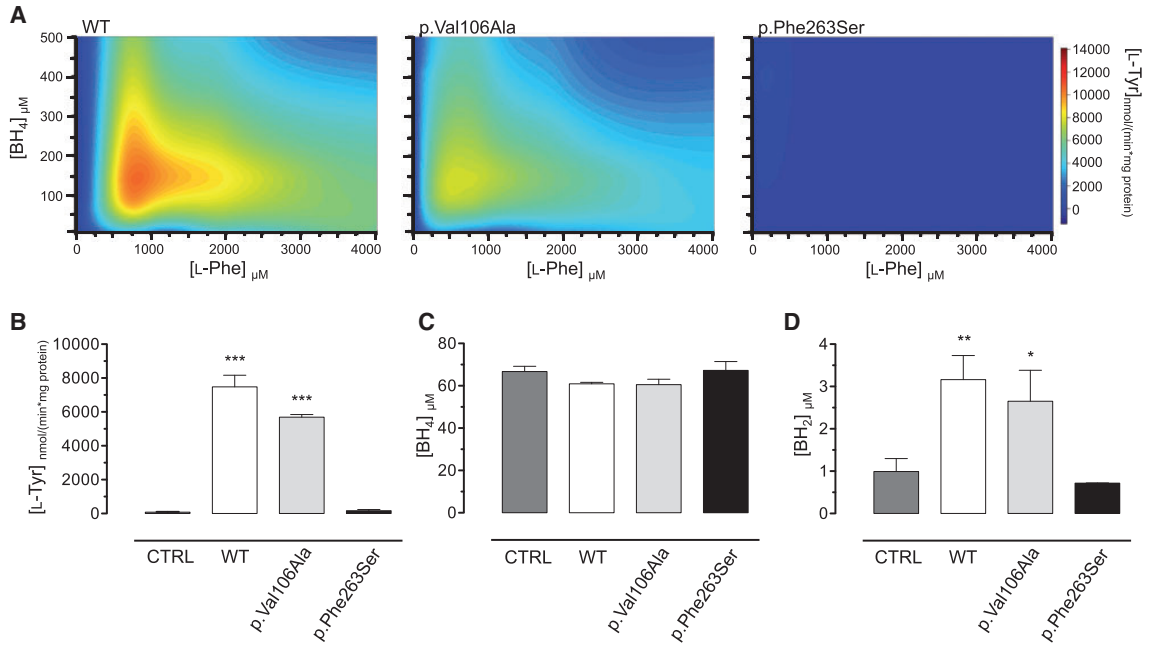

Figure 2. Functional analyses of murine wild-type and the variant Pah enzymes p.Val106Ala and p.Phe263Ser. (**A**) Pah activity landscapes assayed at varying L-Phe (0–4000 µM) and BH_4 (0–500 µM) concentrations. Enzyme activity of purified Pah wild-type and murine variants [L-tyrosine nmol/(min × mg protein)] was color-coded. Each landscape is given as mean of at least three independent experiments. (**B–D**) Enzymatic coupling of L-tyrosine (L-Tyr) and BH_4 for wild-type and variant Pah enzymes. Enzymatic conversion of L-Phe to L-Tyr, BH_4 turnover, and BH_2 production was analyzed by tetrahydrobiopterin assay performed in the samples derived from PAH enzyme activity assays (standard conditions, 1 mM L-Phe and 75 µM BH_4) using recombinant Pah proteins. Data were evaluated using ANOVA with Bonferroni's multiple comparison test. Levels of significance are indicated (*$P \leq 0.05$ **$P \leq 0.01$ ***$P \leq 0.001$). Data are given as means of $n = 3$ independent experiments ± SD.

Table 1. Pah activity landscapes: peak enzyme activity and optimal working range of purified recombinant wild-type and variant Pah

Protein	Peak Pah activity [nmol L-tyrosine/(min × mg protein)]	Activity (% of WT)	L-Phe concentration at peak activity [µM]	$[S]_{0.5} - K_i$ [µM]	BH_4 concentration at peak activity [µM]	$[C]_{0.5} - K_i$ [µM]
WT	10 395	100	892	456–1111	141	45–280
p.Val106Ala	7813	75	561	164–991	137	44–303
p.Phe263Ser	n. a.	n. a.	n. a.	n. a.	n. a.	n. a.

n. a., not applicable; no peak area was delimitable. $[S]_{0.5} - K_i$ indicates the optimal Pah working range (>50% of the respective residual activity) as to substrate concentrations. $[C]_{0.5} - K_i$ indicates the optimal Pah working range (>50% of the respective residual activity) as to cofactor concentrations.

concentration of sepiapterin, hence the precursor was fully converted to BH_4. For all cells expressing murine Pah, the BH_4-concentration was reduced with the lowest concentration observed for p.Val106Ala (30 µM), however these observations did not reach level of statistical significance. After washing off sepiapterin and 4 h incubation without sepiapterin, free BH_4 was overall decreased. The concentration of free BH_4 in cells expressing p.Val106Ala (4 µM) was about 2-fold lower than in cells expressing wild-type Pah (10 µM), which corresponded to the data from isolated hepatocytes (Fig. 1D). Free BH_4 in cells expressing p.Phe263Ser did not differ from wild-type cells (9 µM). Incubation of cells with and without sepiapterin did not have impact on the variant-specific amount of Pah (Supplementary Material, Fig. S1). When BH_4 was analyzed at different time-points after washing off sepiapterin, we did not observe differences in the kinetics of BH_4 decrease (Supplementary Material, Fig. S2).

Therefore, we hypothesized that lower concentrations of BH_4 in isolated hepatocytes or COS-7 cells expressing p.Val106Ala-Pah were due to differences in the ratio of free and bound BH_4. Replacement of valine by alanine at position 106 leads to misfolding of Pah with reduced proteolytic and thermal stability as well as an increase in hydrophobicity and aggregation kinetics (44,54). In order to mirror the misfolding phenotype in Pah^{enu1} hepatocytes, we established conditions where p.Val106Ala is not denatured (Fig. 3B) but partly aggregated (Fig. 3C). Purified wild-type and variant Pah proteins were incubated with equimolar (40 µM) concentrations of BH_4 to analyze pools of unbound BH_4, determined after centrifugation, as well as bound BH_4, after acidic denaturation of Pah (Fig. 3D, Supplementary Material, Table S4). All BH_4 that was added to the no protein control (CTRL1) was recovered after steps of centrifugation and denaturation and there was no cross-contamination of proteins with exogenous BH_4 (CTRL 2). Given

Table 2. Enzyme kinetic parameters at standard L-Phe (1 mM) and BH_4 (75 μM) calculated from activity landscapes

Protein	V_{max} [nmol L-tyrosine/(min x mg protein)]	$[S]_{0.5}$ (L-Phe) [μM][a]	K_M (BH_4) [μM][b]
WT	9584 ± 372 (L-Phe)[a] 9922 ± 490 (BH_4)[b]	227 ± 17	23 ± 2.1
p.Val106Ala	8580 ± 648 (L-Phe)[a] 7121 ± 104 (BH_4)[b]	196 ± 41	24 ± 0.63
p.Phe263Ser	0 (L-Phe)[a] 129 ± 121 (BH_4)[b]	n. d	n. d.

Steady-state kinetic parameters of variant tetrameric MBP-PAH fusion proteins determined by direct in-well activity measurements. V_{max} and the apparent affinities for. L-Phe ($S_{0.5}$) and BH_4 ($C_{0.5}/K_M$) are shown. Values are given as mean ± SD of three or five independent measurements. n.d., not determined.

[a]Enzyme kinetic parameters determined at variable L-Phe concentrations (0–1000 μM) and standard BH_4 concentration (75 μM) with preincubation of the enzyme by L-Phe.

[b]Enzyme kinetic parameters determined at variable BH_4 concentrations (0–125 μM) and standard L-Phe concentration (1 mM) with preincubation of the enzyme by L-Phe (1 mM).

an affinity of ∼24 μM (Table 2), more than half of BH_4 was estimated to be bound to Pah. For the wild-type, the pool of unbound BH_4 was 15.3 and 15.7 μM of bound BH_4 was recovered after denaturation. A minor percentage (7 μM; 19%) was trapped, which is the difference between the applied BH_4 and the pools of bound and recovered BH_4. In contrast, a major share of the pool of bound BH_4 was trapped (14 μM; 35%) by p.Val106Ala and only 3.5 μM were recovered, which is a decrease by 4.5-fold as compared with the wild-type. In addition, the concentration of unbound BH_4 was increased (22 μM). The catalytically inactive variant p.Phe263Ser displayed an elevated pool of unbound BH_4 as compared with the wild-type but the percentage of trapped BH_4 (7.3 μM; 19%) was similar to the wild-type (Supplementary Material, Fig. S4).

Hence, our studies provided evidence that conformational destabilization and aggregation of p.Val106Ala may substantially contribute to secondary BH_4 deficiency observed in $Pah^{enu1/1}$ and $Pah^{enu1/2}$.

Discussion

Tight control of phenylalanine concentrations in body fluids is essential for cognitive development in children and executive function in adults. The phenylalanine hydroxylating system in the liver is rate limiting for disposal of nutrient phenylalanine intake and its function is primarily regulated by the ratio of L-Phe to BH_4. Here we demonstrate that secondary BH_4 deficiency contributes to loss of metabolic function in the phenylalanine pathway and dissect a mechanism how variant-induced perturbation of protein homeostasis interferes with metabolic regulation.

A balanced metabolism of the essential amino acid L-Phe depends on multifaceted *PAH* gene-environment and gene–nutrition interactions. Availability of phenylalanine is mainly governed by nutrient intake. Phenylalanine binding to an N-terminal allosteric site of PAH induces conformational enzyme activation (20,55) but at low L-Phe concentrations, BH_4 acts as inhibitor of PAH to avoid depletion of phenylalanine from body fluids. In liver cells, BH_4 is generally adjusted to equimolarity with the PAH protein (∼10 μM) (14,15); however, treatment of a subset of patients with pharmacological doses of BH_4 subjects blood concentrations to changes of 4- to 10-fold over baseline (36,37,39). Given a K_M of ∼24 μM for BH_4, the PAH enzyme works at suboptimal conditions at physiologic BH_4 concentrations. With increasing phenylalanine concentrations human PAH needs more BH_4 to reach peak activity (24,56). Pah activity landscapes showed that, for maximum phenylalanine turnover, the murine Pah hydroxylating system requires BH_4 concentrations far above the physiologic range. The adaptation to a higher need for BH_4 at elevated blood phenylalanine concentrations in Pah deficient mice occurs by an increase in BH_4 *de novo* synthesis as shown by elevated levels of *Gch1* mRNA. A 10-fold increase in blood phenylalanine for $Pah^{enu2/2}$ resulted in 3-fold higher blood BH_4. However, Pah deficient mice did not adapt liver BH_4 concentrations to elevated blood phenylalanine concentrations. Hepatic BH_4 of animals homozygous for the *enu2* allele was at the wild-type level while BH_4 concentrations were even decreased in animals carrying the *enu1* allele. A correlation of blood BH_4 with blood phenylalanine concentrations has been demonstrated before in $Pah^{enu1/2}$ mice (45); however, a BH_4 decrease in liver homogenates had not been observed, which may be due to methodological differences. With a mean blood volume of 36 ml per 100 g murine liver (57) liver homogenates may contain significant amounts of blood BH_4 and thus explain the differences to BH_4 concentrations of isolated liver cells used in our study. In order to verify our *in vivo* findings in an independent experimental setting, we expressed wild-type and variant murine p.Val106Ala-Pah in COS-7 cells, which were incubated with the BH_4 precursor sepiapterin, and confirmed a lower amount of free BH_4 in p.Val106Ala cells.

We further asked which mechanisms are engaged in secondary BH_4 deficiency. Differences in blood and liver BH_4 of animals carrying the Pah^{enu1} allele suggested a hepatic origin of these findings. For human *PAH* variants, alterations in K_M for BH_4 dependent PAH enzyme activity have been described in (58); however, p.Val106Ala-Pah displayed K_M values similar to the wild-type protein for purified recombinant proteins as wells as upon expression in eukaryotic cells. In order to evaluate the catalytic interaction of Pah, the BH_4 cofactor, and the L-Phe substrate, we used activity landscapes. Pah activity landscapes enabled us to analyze Pah activity as a function of a broad range of BH_4 and L-Phe concentrations (24). We observed comparable optimal working ranges of wild-type, $Pah^{enu1/1}$ and $Pah^{enu1/2}$ in regard to the cofactor concentration. Some *PAH* variants have been reported to be associated with uncoupling of the enzymatic reaction (58). Enzymatic uncoupling leads to an excessive consumption of BH_4 whilst not resulting in an equal amount of tyrosine substrate production (51), eventually leading to an inadequate increase of BH_2. Our experiments ruled out uncoupling for murine p.Val106Ala-Pah, giving therefore no explanation for decreased BH_4 levels in the liver of $Pah^{enu1/1}$ and $Pah^{enu1/2}$. Previous studies described kinetic instability of p.Val106Ala-Pah with proneness to misfolding and aggregation (44,45). Here we used an *in vitro* model

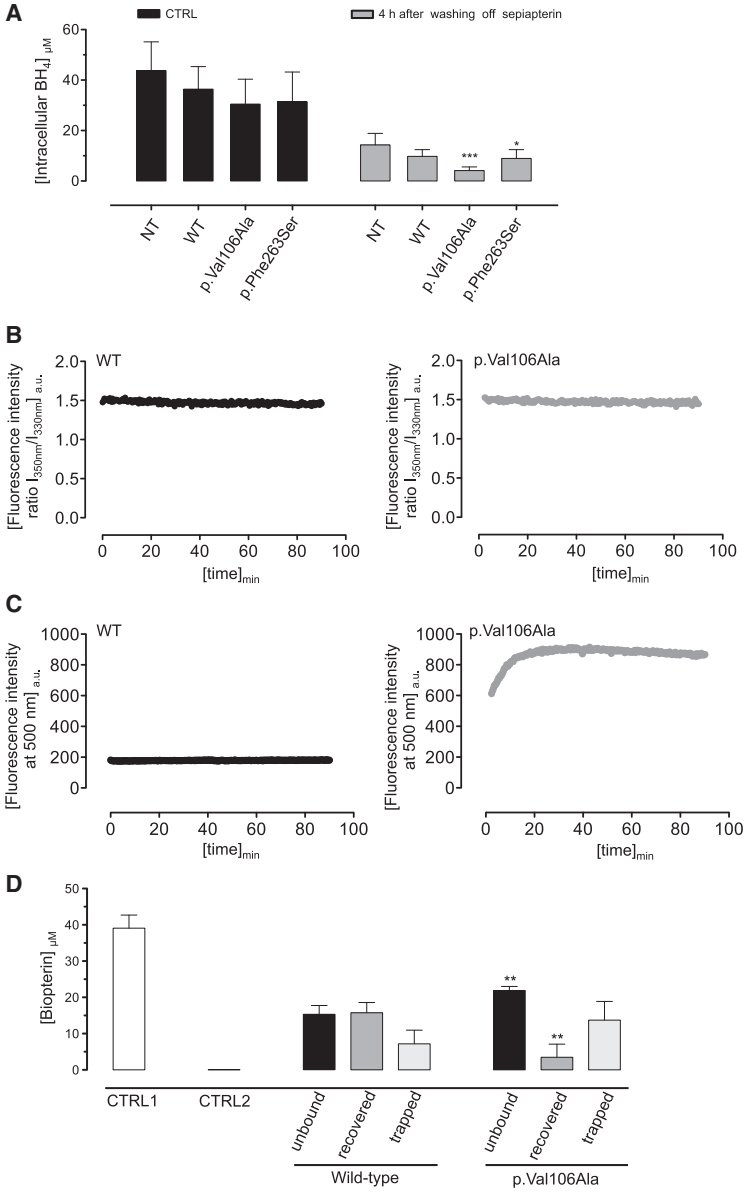

Figure 3. Intracellular BH_4 homeostasis and pools. (**A**) Clearance of intracellular BH_4 after 24 h incubation of transiently transfected COS-7 cells in sepiapterin-enriched (40 µM) medium. BH_4 concentration was determined in cells immediately harvested at the end of the incubation period (black bars) or the residual sepiapterin was washed away and the cells were incubated for further 4 h in standard medium (without sepiapterin) and then harvested (gray bars). Values are given as mean of $n = 3$ independent experiments ± SD. Aggregation kinetics of purified murine Pah determined by intrinsic tryptophan fluorescence. (**B**) Fluorescence intensity ratio (intensity recorded at emission wavelength of 350 nm/intensity at emission wavelength of 300 nm) showing unfolding of the wild-type and p.Val106Ala over time (**C**) Fluorescence intensity recorded at emission wavelength of 500 nm describing aggregation events for the wild-type and p.Val106Ala over time. (**D**) Pools of bound and unbound BH_4 identified *in vitro* in purified wild-type (WT) and p.Val106Ala proteins. *Escherichia coli* purified Pah enzymes were incubated with equimolar amounts of BH_4 followed by a four-step centrifugation to discriminate different pools of BH_4, Pah-unbound BH_4 (unbound, black bars), recovered Pah-bound BH_4 (recovered, dark gray bars), unrecovered Pah-bound BH_4 (trapped, light gray bars). Control 1 (CTRL 1), biopterin recovery of 40 µM BH_4 in a PAH-free buffer. Control 2 (CTRL 2), biopterin recovery of a BH_4-free buffer containing 40 µM wild-type Pah. Values are given as mean of $n = 3$ independent experiments ± SD. Significant differences compared to WT are indicated (**$P \leq 0.01$).

where purified p.Val106Ala was partially aggregated but not unfolded while wild-type Pah maintained a native-like conformation, as shown by intrinsic tryptophan fluorescence. Upon complete denaturation, the wild-type protein released the major share of bound BH_4, whereas BH_4 was trapped by p.Val106Ala. The misfolded variant Pah shifted the ratio of unbound to bound BH_4 and therefore induced a loss of free BH_4 in livers of $Pah^{enu1/1}$ and $Pah^{enu1/2}$ animals.

An imbalance in the ratio of L-Phe and BH_4 due to secondary BH_4 deficiency might interfere with the metabolite controlled regulation of the phenylalanine hydroxylating system in humans. In patients with PAH deficiency, trapping of BH_4 by misfolded variant PAH may lead to a decrease in free BH_4 and therefore reduce the pool of BH_4 available for catalytic degradation of L-Phe. Thus, differences in the need for BH_4 in PKU patients may, at least in part, be driven by genotype-specific and protein misfolding-induced alterations of free hepatic BH_4. Moreover, trapping of BH_4 by misfolded variant PAH may interfere with pools of pharmacologically administered BH_4. Hence, our observations may help to optimize strategies to identify BH_4-responsive patients as well as to implement genotype-specific dosing in BH_4 treatment.

We raised the question whether the observed secondary BH_4 deficiency may affect function and dysfunction of related metabolic pathways. BH_4 plays a centrol role in degradation of aromatic amino acids and is an essential cofactor for PAH, tyrosine hydroxylase and tryptophan hydroxylase, enzymes which are key in the metabolism of neurotransmitters. In addition, BH_4 is an essential cofactor for endothelial nitric oxide synthase (eNOS). Enzyme function depends on cofactor availability (59,60) and decreased BH_4 availability results in eNOS uncoupling with enhanced production of superoxide instead of nitric oxide (61). Uncoupling was also reported for conditions of increased BH_4 oxidation (62). For patients suffering from severe malaria (63), increased conversion of BH_4 to BH_2 due to increased oxidative stress and insufficient recycling of BH_2 back to BH_4 has been demonstrated.

In the cell, protein unfolding is a frequent event and protein homeostasis is challenged by general physiological perturbations such as oxidative stress or fever. Intricate networks of molecular chaperones and the ubiquitin-proteasome system are responsible for homeostasis of client proteins (64–66), moreover, chaperones mediate degradation of metabolites (67). In case the cytosolic proteostasis network is unable to handle the variant-induced perturbation of the PAH protein folding energy landscape, energetically trapped misfolded states will accumulate and misfolded protein species will be subjected to the degradation pathway. On the other hand, proteostasis of PAH depends on availability of BH_4 (45). Dysregulation of PAH proteostasis due to a deficiency in the molecular chaperone DNAJC12 was recently reported, interestingly, this defect could partially be corrected by pharmacological doses of BH_4 (68).

In this study, we dissect a mechanism how variant-induced perturbation of protein homeostasis interferes with metabolic regulation. Expanding the insight into the complex nature of gene–nutrition and gene–drug interactions will contribute to our knowledge of metabolic pathway architecture and of the adaptation of metabolism to health and disease.

Materials and methods

Plasmid construction

The cDNA of murine *Pah* (EST clone from imaGene) was cloned into the pcDNA3.1/myc-His C vector (life technologies). Substitutions resulting in murine variants p.Val106Ala and p.Phe263Ser were introduced into the pcDNA3.1 plasmid using the PCR-based QuikChange site-directed mutagenesis kit (Stratagene) and authenticity was verified by DNA sequencing. Subsequently, wild-type and mutant cDNAs were subcloned into the prokaryotic expression vector pMAL-c2E (New England Biolabs) and a Gateway-modified pMAL-c2x vector with an additional TEV cleavage site. For eukaryotic expression of wild-type and variant Pah, the pEF-DEST51 vector (life technologies) was used. Murine genes were amplified using primers containing flanking *att*B sites and the Kozak sequence at the 5′ terminus. PCR products were subsequently cloned into the pDONR221 vector (life technologies) followed by subcloning to pEF-DEST51 (life technologies).

Animals

Wild-type (BTBR $T^{+}tf/J$), $Pah^{enu1/1}$ (BTBR.Cg-Pah^{enu1}/J), and $Pah^{enu2/2}$ (BTBR-Pah^{enu2}/J) mice were purchased from The Jackson Laboratory (Bar Harbor, USA). The compound heterozygous hybrids $Pah^{enu1/2}$ were crossbred in our animal facility. Animals were housed under controlled temperature conditions and maintained on a cycle of 12-h light/dark period. Between experiments water and food were available *ad libitum*. Tests were carried out in adult animals at 2–10 months of age. All animal experiments were performed in accordance with German animal protection law.

Measurement of blood phenylalanine levels

Prior determination of blood L-Phe concentrations, mice were kept on normal diet. Blood samples were collected and spotted onto the filter cards immediately after the mice were sacrificed. The analysis was performed via electrospray ionization tandem mass spectrometry in the laboratory of R. Fingerhut (Kinderspital Zurich).

Isolation of murine hepatocytes

The hepatocytes were harvested according to an existing protocol (69) with minor changes. After isoflurane narcosis and cervical dislocation, the mouse was fixed on a heating plate (39°C). The liver was exposed and a perfusion system through the right atrium was established. Three perfusion steps were started with a flow of 5 ml/min; 4 min with ethylene glycol-bis(β-aminoethyl ether)-N,N,N′,N′-tetraacetic acid (EGTA) buffer (0.1× Leffert's buffer with 0.5 mM EGTA), 2 min with 1× Leffert's buffer (10 mM HEPES, 3 mM KCl, 130 mM NaCl, 1.2 mM NaH_2PO_4, 10 mM D-Glucose) and 20 min with collagenase buffer (1× Leffert's buffer with 2.5 mM calcium chloride and 40 mg/l collagenase). The murine cells were extracted from the liver capsule with a cell scraper. The cell suspension was filtered using three cell strainers with a pore width of 100 μm. The solution was centrifuged (3 min at 50*g*, 4°C) and resuspended in 30 ml hepatocyte wash medium (invitrogen). Following centrifugation at 50*g*, 4°C for 1 min, an aliquot of the cell pellet was resuspended in modified Williams E Medium (invitrogen). Cell viability and the cell amount were evaluated using trypan blue staining (life technologies) and a Neubauer counting chamber (blaubrand). The cell pellet was resuspended in 200 μl lysis buffer [0.2 M KCl, 0.03 M Tris, pH 7.2 containing protease inhibitor (Roche)] and underwent three freeze (−80°C) and thaw (37°C) cycles for cell lysis. The cell suspension was centrifuged in two steps (5 min at 1000*g* and 15 min at 20 000*g*, 4°C) and the supernatant was frozen in aliquots in liquid nitrogen for further use. All buffers were prepared as stock solutions, adjusted to a pH of 7.4, filtered and stored at 4°C.

Quantitative real-time PCR

Total RNA was extracted from isolated murine hepatocytes (2 million cells), which were not lysed after isolation. The

contaminating DNA was removed with the help of Masterpure RNA Purification Kit (Epicentre Biotechnologies). Reverse transcription was performed using the QuantiTect Reverse Transcription Kit (Qiagen) and the DNA was stored at –20°C until the real-time PCR was performed. Quantitative PCR was performed with cDNA on a Step One Plus real-time PCR system (Applied Biosystems) using Power SYBR Green PCR Master Mix (Applied Biosystems). Gene expression levels of murine *Gch1* were normalized to the housekeeping gene murine *Gapdh*. β2-*microglobulin* was used as a positive control. Primers designed for mRNA transcripts are summarized in Supplementary Material, Table S5.

Protein expression and purification

Escherichia coli DH5α cells were transformed with expression plasmids coding for murine wild-type and variant Pah, which were N-terminally fused to maltose-binding protein (MBP). Recombinant proteins were overexpressed for 20 h at 37°C. and purified as previously described in (12,44). For TEV cleaved Pah, affinity purified proteins were incubated overnight at 4°C with TEV protease followed by size exclusion chromatography using ÄKTApurifier (GE Healthcare). The concentration of the isolated tetramers was determined spectrophotometrically with A_{280} (1 mg/ml) = 1.63 for MBP-Pah and $A_{280\ nm}$ (1 mg/ml) = 1 for Pah. sodium dodecylsulfate-polyacrylamide gel electrophoresis and Coomassie staining verified homogeneity of purified proteins.

Intrinsic tryptophan fluorescence and right angle light scattering

Intrinsic tryptophan fluorescence and right angle light scattering (RALS) measurements were performed using a Cary Eclipse spectrofluorimeter equipped with a temperature-controlled Peltier multicell holder (Varian). MBP-Pah fusion proteins of the wild-type and the variant p.Val106Ala were analyzed. To monitor the folded and unfolded states of Pah, intrinsic tryptophan fluorescence emission spectra were recorded with excitation at 295 nm; folded and unfolded state of PAH were analyzed at 300 and 350 nm (70). For unfolding kinetics of wild-type and p.Val106Ala, tryptophan fluorescence intensity of 1 μM protein at 300 and 350 nm (slit width 7 nm) was recorded at 25°C. Data are given as the ratio of fluorescence intensity at 350–300 nm representing the ratio of the unfolded state to the folded state. Additionally, for aggregation kinetics of MBB-Pah wild-type and p.Val106Ala RALS was monitored at 37°C for 90 min (excitation and emission wavelength of 500 nm).

Transient expression of murine Pah in COS-7

COS-7 cells were maintained and transient transfection with pEF-DEST51 cDNA was performed as previously described in (44). 4 h after transfection the media were supplemented with 40 μM sepiapterin stabilized in DTT (final concentration, Schircks Laboratories) and the cells were further cultivated for 24 h. To analyze the intracellular BH_4 content after exposure of the cells to sepiapterin the cells were washed twice with PBS (gibco by life technologies) to remove the residual compound and cells were immediately lysed (direct control of accumulated BH_4 in the cells) or cultivated for additional 1, 2, 4, 6 and 8 h in standard RPMI 1640 media (time-dependent analysis of BH_4 content in the cells after BH_4 removal from the environment). Cells were harvested and lysed by three freeze-thaw cycles (44); 40 μl of cell lysate was used in the biopterin assay. Not transfected cells served as an additional control.

PAH enzyme activity assays

PAH enzyme activity in primary hepatocytes was determined as previously described with modifications (26). 20 μl of the cell lysates were preincubated with 1 mM L-Phe and 1 mg/ml catalase for 5 min at 25°C in 15 mM Na HEPES pH 7.3, followed by 1 min incubation with 10 μM ferrous ammonium sulfate. The reaction was initiated by the addition of 75 μM BH_4 stabilized in 2 mM dithiothreitol (DTT, Sigma-Aldrich), carried out for 60 min at 25°C and stopped by acetic acid followed by 10 min incubation at 95°C. In case of PAH activity assay performed with tetrameric wild-type and variant MBP-Pah 20 μl of 0.01 mg/ml protein was used in the reaction mixture and the reaction was carried out for 1 min. All concentrations mentioned refer to the final concentration in a 100 μl reaction mixture. The amount of L-tyrosine production was measured and quantified by HPLC, assayed as duplicates or triplicates.

To analyze BH_4 usage and BH_2 production during the reaction catalyzed by purified murine wild-type and variant MBP-Pah, the standard PAH activity assay was performed with modifications. The reaction was carried out for 1 min and stopped with 8 M urea to assure protein denaturation and prevent biopterin from undue destruction. In total 40 μl of the reaction mixture was then used in the biopterin assay. As a control, the PAH activity assay was performed without Pah protein.

The multi-well PAH activity assay for determination of activity landscapes using varying concentrations of BH_4 (0–500 μM) and L-Phe (0–4000 μM) was performed and bioinformatically analyzed as previously described in (24). All PAH activity landscapes were assayed in three to five independent experiments and data were combined.

BH_4 trapping assay

To determine the pools of bound and unbound BH_4 a trapping assay was performed using murine wild-type, p.Val106Ala and p.Phe263Ser MBP-Pah as well as TEV-cleaved murine wild-type and p.Phe263Ser. Equimolar amounts of BH_4 and Pah (40 μM) were incubated for 15 min at room temperature. The mixtures were then transferred to Amicon ultra centrifugal filters (Millipore) and centrifuged for 8 min at 3200 rpm (Hettich Rotana 460R 5624), 4°C. The resulting flowthrough was kept for the analysis of BH_4 content (unbound BH_4). The proteins from the upper reservoir were washed three times with 1 ml size-exclusion buffer (200 mM NaCl and 20 mM HEPES, pH 7.0). To analyze the amount of BH_4 released from the protein (recovered BH_4), murine Pah proteins were subjected to 5% TCA and after 30 min incubation time mixtures were centrifuged at 20 000*g*, 10 min at 4°C. Next, 40 μl of the flowthrough samples as well as TCA-treated samples were used in the biopterin assay. As internal assay controls, samples of native Pah (without BH_4) and BH_4 (without Pah) were used. The amount of trapped BH_4 was calculated by subtracting unbound and released BH_4 from the whole amount of the BH_4 measured in the assay. Trapping assay was performed as three independent experiments.

Biopterin assay

Total biopterin concentration was analyzed by HPLC according to established methods (14,71). An aliquot of 40 μl of the sample

was mixed with 50 μl of an acidic- (1% I_2/2% KI in 0.1 M HCl) or alkaline-iodine solution (1% I_2/2% KI in 0.2 M NaOH) and incubated for 1 h in the dark at room temperature. The reaction was stopped by 20 mM (final concentration) of ascorbic acid (for the acidic reaction) or 20 mM ascorbic acid in HCl (alkaline reaction) followed by centrifugation at 10 000*g* for 20 min. Biopterin was determined by HPLC equipped with a fluorescence detector ($E_x = 350$ and $E_m = 450$ nm). The amount of tetrahydrobiopterin was determined by calculating the difference of biopterin measured in acidic conditions and biopterin measured in alkaline conditions (36).

Statistical analyses

One-way analysis of variance (ANOVA) with Dunnett's or Bonferroni's *post hoc* test was applied for multiple comparisons. Statistical analyses were performed using GraphPad Prism 5.0 (GraphPad Software).

Supplementary Material

Supplementary Material is available at *HMG* online.

Conflict of Interest statement. A.C.M. is member of SAB of BioMarin and Nutricia.

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