Human tyrosine hydroxylase: Oxygen dependence and role in Dopa responsive dystonia

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Scientific environment

The work presented in this thesis was carried out at the Department of Biomedicine, University of Bergen, full-time during the period October 2004 – October 2005, and February 2011-April 2013, and part time 2013- 2015; under the supervision of Professor Jan Haavik. Professor Aurora Martinez was co-supervisor during the PhD fellowship from 2011-2015. The Faculty of Medicine and Dentistry at the University of Bergen provided financial support for “Forskerlinje” and PhD fellowships.

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Abbreviations

5-HT, 5-hydroxytryptamine, serotonin

AAH, aromatic amino acid hydroxylases

ADHD, attention deficit hyperactivity disorder

BBB, blood brain barrier

BH_4, (6R)-L-erythro-5,6,7,8-tetrahydrobiopterin

CaMKII, calcium/calmodulin dependent protein kinase

CNS, central nervous system

CSAS, central sleep apnoea syndrome

CSF, cerebrospinal fluid

HPA, hyperphenylalaninemia

IH, intermittent hypoxia

L-DOPA, 3,4-dihydroxyphenylalanine

OSAS, obstructive sleep apnoea syndrome

PAH, phenylalanine hydroxylase

PD, Parkinson’s disease

PDB, protein databank

PKA, cAMP-dependent protein kinase

PKU, phenylketonuria

ROS, reactive oxygen species

TH, tyrosine hydroxylase

THD, tyrosine hydroxylase deficiency

TPH, tryptophan hydroxylase

WT, wild type

Abstract

The aromatic amino acid hydroxylases (AAHs); phenylalanine hydroxylase (PAH), tyrosine hydroxylase (TH), tryptophan hydroxylase 1 and 2 (TPH 1/TPH 2); are structurally and functionally related enzymes. All AAHs require iron, dioxygen and the cofactor (6R)-L-erythro-5,6,7,8-tetrahydrobiopterin (BH_4) to be catalytically active. PAH is the first and rate-limiting enzyme in the catabolism of L-Phe; TH is the rate-limiting enzyme in the synthesis of the catecholamine neurotransmitters dopamine, adrenaline and noradrenaline; while the TPH 1 and 2 catalyses the first and rate-limiting reaction in then synthesis of serotonin and melatonin.

These physiologically important enzymes are implicated in human diseases; polymorphisms and variants in the TPH genes are associated to neuropsychiatric disorders, mutations in PAH and TH are responsible for the autosomal recessive disorders phenylketonuria (PKU) and TH deficiency (THD), respectively. Furthermore, a role of TH in Parkinson's disease and hypoxia induced diseases, such as altitude sickness and sleep apnoea, has been suggested.

The aims of this project were to 1) develop improved methods of studying AAHs, mainly focusing on their dependence of oxygen; 2) investigate the role of oxygen in the AAH reactions, with an emphasis on TH; 3) to characterize the effect of mutations in AAH, mainly PAH and TH, on their enzyme function and stability; 4) investigate the effects of missense *TH* mutations reported in patients with THD and perform genotype-phenotype comparisons in these patients.

The thesis is based on three separate articles (Article 1-3); the first article is focused on developing a new oxygrapic assay method for studying the activity and enzymatic mechanism of AAHs by monitoring the oxygen consumption continuously during the catalytic reaction. The second paper describes the oxygen dependence of TH in normoxic and hypoxic conditions, relevant for physiological effects of high altitude and other conditions of low oxygen availability. In the third article, mutated variants of TH reported in patients with THD, were characterized and compared to wild-type (wt)-TH with regards to *in vitro* solubility, thermal stability and kinetic properties.

In Article 1 we demonstrated the utility of a new oxygrapic assay to study the function of AAHs. We studied kinetic properties and enzyme reaction mechanisms of both wt and mutant enzyme using different substrates and cofactors. A stable reaction stoichiometry of 1:1 was obtained between the amount of oxygen consumed and tyrosine formation when the natural cofactor (6R)-tetrahydrobiopterin was added as electron donor in the phenylalanine hydroxylase (PAH) reaction. In comparison, low and variable coupling efficiency values between oxygen consumption and tyrosine formation were found using the parent unsubstituted tetrahydropterin. Furthermore, we studied the phenylketonuria-associated PAH mutant R158Q and found that the reaction had a coupling efficiency of about 80 % compared to the wild-type enzyme under similar conditions. The high time resolution of this method allowed us to obtain new knowledge about the initial reaction kinetics of the AAHs.

These findings were investigated further in Article 2; where we observed an initial high activity phase in the first 1-2 minutes of the TH reaction, levelling off to a lower stable activity rate after the initial phase. During the initial reaction phase, apparent K_m-values of 29–45 μM for dioxygen were determined for all human TH isoforms, i.e. 2–40 times higher than previously reported for TH isolated from animal tissues. After 8 min incubation, the K_m (O_2)-values had declined to an average of 20 ± 4 μM.

In Article 3, 22 different missense and one nonsense coding variants from patients with THD were produced in *E. coli* and subjected to biochemical studies of their enzymatic properties. Compared to wt-TH we observed a great heterogeneity of changes in the *in vitro* solubility, thermal stability and enzymatic activity among the mutated TH variants; indicating different pathogenetic mechanisms of the TH mutations found in patients with THD.

In conclusion, this project has established the new oxygraphic method as a valuable supplement to other activity assays of AAHs, providing an assay which is versatile, fairly sensitive and has a high time resolution. New insights into the initial phase of the enzymatic reaction of the AAHs has revealed a previously undescribed shift to a low activity phase of the enzymes *in vitro*, which may be due to a rate limiting regeneration

of the active site iron from the inactive ferric form in the catalytic cycle. This may be significant in understanding the physiological effect of hypoxic conditions, as the concentration of oxygen in tissues reaches the K_m-values for TH calculated in this study. Characterization of THD associated variants of mutated TH have increased the understanding of the molecular mechanisms of their pathogenicity; contributing to the understanding of the neurological symptoms and complementing the animal and clinical studies conducted to develop new and personalized treatments for THD patients.

List of publications

I. Fossbakk, A. & Haavik, J. (2005): “An oxygraphic method for determining kinetic properties and catalytic mechanism of aromatic amino acid hydroxylases”, Analytical Biochemistry 343 (2005) 100-105.

II. Rostrup, M, Fossbakk, A, Hauge, A, Kleppe, R, Gnaiger, E and Haavik, J. (2008): “Oxygen dependence of tyrosine hydroxylase”, Amino Acids 34: 455-464.

III. Fossbakk, A., Kleppe, R., Knappskog, P.M., Martinez, A. & Haavik, J. (2014): “Functional studies of tyrosine hydroxylase missense variants reveal distinct patterns of molecular defects in DOPA responsive dystonia”, Human Mutation 35: 7, 880-890.

The following related articles are also referred to in the text, but are not part of this thesis:

Regulation of tyrosine hydroxylase is preserved across different homo- and heterodimeric 14-3-3 proteins.
Ghorbani S, Fossbakk A, Jorge-Finnigan A, Flydal MI, Haavik J, Kleppe R.
Amino Acids. 2016 Jan 29. [Epub ahead of print]

Effects of Missense Mutations in Tyrosine Hydroxylase (TH) Found in Patients with Neurological Disorders Attributed to TH Deficiency, Page 25, Agnete Fossbakk, Per M. Knappskog, Aurora Martinez, Jan Haavik. Catecholamine Research in the 21st Century . 2013 Edited by: Lee E. Eiden ISBN: 978-0-12-800044 p. 25.

Modeling the Dynamics of Dopamine Biosynthesis and its Regulation by Tyrosine Hydroxylase, Page 30, Rune Kleppe, Sadaf Ghorbani, Agnete Fossbakk, Aurora Martinez, Jan Haavik. Catecholamine Research in the 21st Century. 2013 Edited by: Lee E. Eiden ISBN: 978-0-12-800044 p. 30.

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1. Human tyrosine hydroxylase: Oxygen dependence and role in Dopa responsive dystonia

1.1 Introduction

Tyrosine hydroxylase (TH) is a member of the enzyme family of aromatic amino acid hydroxylases (AAH). The first AAH to be isolated was phenylalanine hydroxylase (PAH) in 1957; its function had already been demonstrated by transformation of labelled phenylalanine to tyrosine in water-soluble liver extracts (Kaufman, 1958). In 1964, the first reports of TH activity in neural tissues were published (Kaufman, 1964; Nagatsu et al., 1964c) and shortly thereafter tryptophan hydroxylase (TPH) (Grahame-Smith, 1964; Lovenberg et al., 1967). The requirement of PAH for a tetrahydropterin cofactor was shown using the synthetic cofactor analogues 6,7-dimethyltetrahydropterin and 6-methyltetrahydropterin (Kaufman and Levenberg, 1959). In 1963, the natural cofactor was identified as tetrahydrobiopterin (BH_4), as this compound was isolated from rat liver extracts (Kaufman, 1963).

1.1.1 General introduction to the aromatic amino acid hydroxylases (AAHs)

The AAHs tyrosine hydroxylase (TH; MIM #191290), phenylalanine hydroxylase (PAH; MIM #612349) and tryptophan hydroxylase 1 and 2 (TPH1; MIM #191060, TPH2; MIM #607478), are considered to be homologous enzymes in structure and function. A common ancestry for the enzymes in this family have been proposed (Ledley et al., 1987; Siltberg-Liberles et al., 2008), as they have approximately 60 % DNA sequence identity and 85 % amino acid sequence identity in their catalytic domains.

All AAH contain a single atom of non-heme iron per enzyme subunit; they require BH_4 as cofactor and use molecular oxygen to hydroxylate their amino acid substrates. The reaction stoichiometry has been found to be 1:1 for all substrates and products formed (Fig. 1). The active site iron is coordinated by two histidine residues and one glutamate as ligands to the iron in all three hydroxylases, and the iron has to be in the ferrous (II) redox state for the enzyme to be active (Costas et al., 2004; Flatmark and Stevens, 1999; Teigen et al., 2007).

Aromatic amino acid + tetrahydropteridine + O_2 → hydroxylated amino acid + dihydropteridine + H_2O

Fig. 1. General reaction of the aromatic amino acid hydroxylases

The normal reaction stoichiometry is dependent on optimal positioning of the substrates in the active site of the enzyme. Using alternative substrates or changing the geometry of the active site can alter the stoichiometry and cause a partially or fully "uncoupled" reaction to take place. In an "uncoupled" reaction more substrate is consumed than product formed (Kappock and Caradonna, 1996). In reactions where more oxygen is consumed than product formed, the excess oxygen may be released as reactive oxygen species (ROS). ROS *in vivo* are potentially harmful to cells.

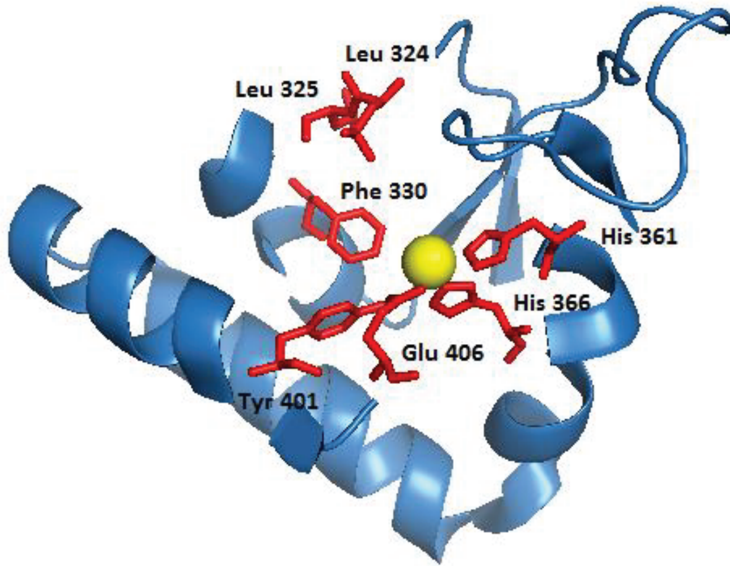
1.1.2 Structure of the aromatic amino acid hydroxylases

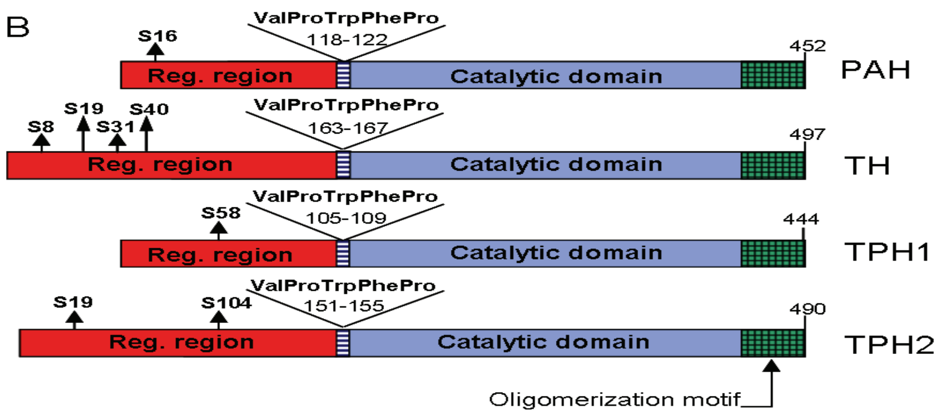
The AAHs fold in similar tertiary structures, with an amino-terminal regulatory domain (100-160 amino acid residues), a catalytic domain (approx. 300 residues) and a tetramerization domain at the carboxyl terminus (approx. 40 residues). All four enzymes form homotetramers in solution. The regulatory domains differ between the AAHs, with only 15 % sequence identity (Daubner et al., 1997), and are also the domains that interact with kinases, phosphatases and binding proteins, like 14-3-3 and α-synuclein (Alerte et al., 2008; Khan et al., 2012; Kleppe et al., 2014; Lou et al., 2010; Skjevik et al., 2014). The common part of the regulatory domains contains an ACT fold that can serve as a sensor modulating allosteric responses to amino acids (Arturo et al., 2016; Kobe et al., 1997). All of the residues required for activity and substrate binding are located within the catalytic domain (Daubner et al., 1993; Moran et al., 1998). Crystal structures are available of the catalytic domains of human (pdb file 1PAH) (Erlandsen et al., 1997) and rat (pdb file 2PHM) (Kobe et al., 1997) PAH; rat (pdb file 1TOH) (Goodwill et al., 1997) and human (pdb file 2XSN) (Muniz et al. 2010) TH; human (pdb file 1MLW) (Cianchetta et al., 2010; Wang et al., 2002) and chicken (pdb file 3E2T) (Windahl et al., 2008) TPH 1, and human TPH 2 (pdb file 4V06) (Kopec et al. 2014) establishing their common structure. Recently, a structure of full-

length tetrameric rat PAH in its inactivated form was published (pdb file 5DEN) (Arturo et al., 2016).

For PAH, a crystal structure with both BH_4 and an amino acid bound to the catalytic domain has been produced (Andersen et al., 2003), and while there is no available structure of either TPH or TH with both BH_4 and an amino acid substrate bound together, there are separate structures of chicken TPH with tryptophan bound and human TPH with dihydrobiopterin bound (Wang et al., 2002; Windahl et al., 2008). These structures make it possible to describe the interactions of all AAH with substrates. BH_4 interacts with the side chains of a glutamate and a phenylalanine residue; the remaining interactions are with backbone atoms. The carboxylate of the amino acid substrate interacts with an arginine and an aspartate residue. The side chain of the amino acid substrate is held in a hydrophobic pocket made up of a proline, a histidine that is also a metal ligand, a phenylalanine, and either a phenylalanine in TPH or a tryptophan in TH and PAH (Almas et al., 2000; Andersen et al., 2001; Andersen et al., 2002; McKinney et al., 2001; Teigen et al., 2007; Wang et al., 2002).

A.





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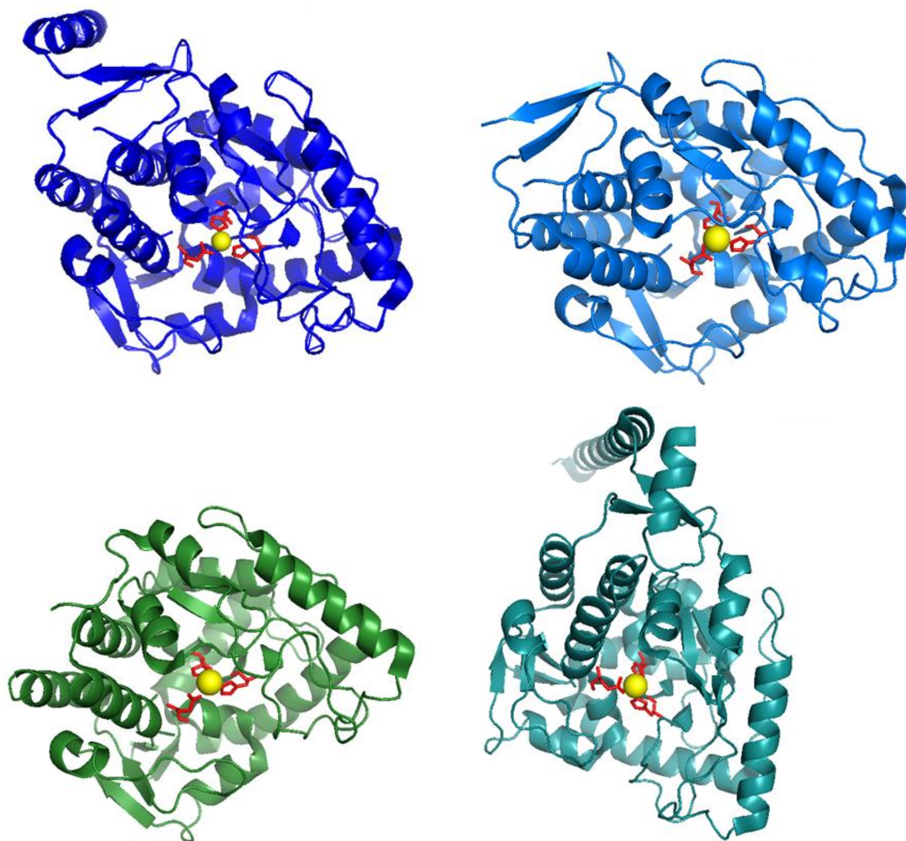

Fig. 2. Structure of the AAHs.

A. Detail of hTH active site with bound Zn^{2+} (PDB 2XSN) and selected residues
B. Schematic illustration of the domain organization of the AAHs; the regulatory N-terminal domain (red) containing one or more serine phosphorylation sites, catalytic domain (blue), and oligomerization domain (green).
C. Ribbon diagrams of the catalytic and tetramerization domains of human tyrosine hydroxylase (upper left, PDB 2XSN), phenylalanine hydroxylase (upper right, 4ANP), tryptophan hydroxylase 1 (lower left, 1MLW) and tryptophan hydroxylase 2 (lower right, 4V06).

1.1.3 Reaction mechanisms of aromatic amino acid hydroxylases with emphasis on tyrosine hydroxylase

Based on steady-state kinetic studies of the recombinant rat TH catalyzed reaction, a sequential reaction mechanism has been proposed, with an ordered binding of tetrahydrobiopterin (BH_4), dioxygen and finally tyrosine (Tyr) (Fitzpatrick, 1991). The binding of BH_4 to TH results in the closing of a loop over the active site; possibly converting the amino acid binding site to the active form (Sura et al., 2006). All studies of the hydroxylases are consistent with a common reaction mechanism (Fitzpatrick, 2003); first the reaction of the BH_4, oxygen and the active site iron to form the reactive hydroxylating intermediate and then the insertion of the oxygen into the amino acid. The partial reactions are tightly coupled. Without the tetrahydropterin and the amino acid bound, the enzyme will not react productively with the oxygen and instead the iron is oxidized to the ferric inactive form (Chow et al., 2009; Frantom et al., 2006). The catalytic activity of TH requires the binding of tyrosine, BH_4 and molecular oxygen to the catalytic site that harbors the ferrous iron coordinated by the side chains of two histidines and a glutamate (Fig. 2 A.). Studies of the enzyme by both X-ray crystallography and spectroscopic techniques have shown that the tyrosine and BH_4 do not bind directly to the iron; rather, binding of tyrosine and BH_4 leads to structural changes that result in the metal center transitioning from 6- to 5- coordinate (Chow et al., 2009). These changes in the catalytic site increase the oxygen reactivity with the iron > 100-fold, and thus trigger the start of a two-step reaction mechanism (Chow et al., 2009; Fitzpatrick et al., 2003).

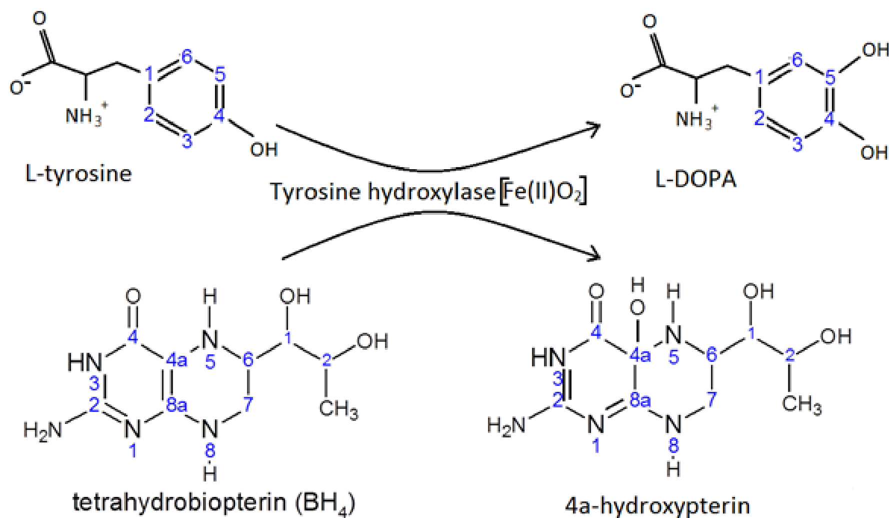

Fig. 3 Reaction of tyrosine hydroxylase

The first step of the TH reaction is the hydroxylation of the 4a-carbon to yield 4a-hydroxy(tetrahydro)pterin. In this step, the BH_4 donates two electrons for the heterolytic cleavage of the O-O bond and leaves a Fe(IV)-oxo intermediate. The Fe(IV)-oxo species have been trapped for TH and characterized by Mössbauer spectroscopy (Eser et al., 2007). The second step of the catalytic mechanism involves attack of the Fe(IV)-oxo species on the phenol side chain of tyrosine to produce L-DOPA by electrophilic aromatic substitution (Eser et al., 2007; Frantom et al., 2006; Hillas and Fitzpatrick, 1996). Tyrosine hydroxylase is a stereospecific enzyme for both substrate and cofactor (Bailey and Ayling, 1978). Partial uncoupling of the enzyme reaction has been observed with several amino acid and cofactor analogues.

1.1.4 Physiological aspects of the aromatic amino acid hydroxylases

PAH is mainly expressed in the liver and kidney, but minor amounts are also found in in melanocytes (Schallreuter et al., 2004), where epidermal PAH produces L-Tyr from the essential amino acid L-Phe, supporting melanogenesis. In liver, the hydroxylation of L-Phe to L-Tyr is the first and rate-limiting step in the catabolism of phenylalanine, resulting in the formation of one molecule of fumarate and one of acetoacetate (Acetyl-CoA). PAH activity is highly regulated to prevent depletion of L-Phe, which is an essential amino acid required for protein synthesis, but also to prevent the excess of

this neurotoxic amino acid. Dysfunction in PAH leading to hyperphenylalaninemia and PKU is described in further detail below.

In rats, 20-45 % of the liver PAH is believed to be in an inactive state (Shiman et al., 1982), and the authors of that study made the point that there is enough PAH present in the liver to deplete the serum of phenylalanine in a few minutes if all PAH was to become fully activated. The PAH activity is regulated by different mechanisms, and physiologically glucagon is known to indirectly stimulate the formation of tyrosine by PAH. PAH is allosterically activated by its substrate phenylalanine, which binds with positive cooperativity to the enzyme. The mechanism of activation is not fully understood, and there are conflicting opinions concerning the site of cooperative binding of L-Phe (Fitzpatrick, 2012; Flydal and Martinez, 2013). There is evidence for L-Phe binding to a specific site in the regulatory domain of PAH, the ACT domain, causing a conformational change and dimerization of the domains (Gjetting et al., 2001; Jaffe et al., 2013; Zhang and Fitzpatrick, 2016), but other studies have found that phenylalanine causes the conformational change and subsequent activation of PAH upon binding to the catalytic site through a homotropic interaction between different subunits (Martinez et al., 1990; Martinez et al., 1993; Thorolfsson et al., 2002; Thorolfsson et al., 2003). On the other hand, the natural cofactor BH_4 negatively regulates PAH activity besides being essential in catalysis. Furthermore, phosphorylation of PAH at Ser16 increases the activity of PAH by reducing the K_d for L-Phe activation (Doskeland et al., 1996).

TH is the rate-limiting enzyme in the synthesis of dopamine, adrenaline and noradrenaline, i.e. the catecholamine neurotransmitters. TH is mainly expressed in the dopaminergic neurons of the ventral tegmental area and *substantia nigra pars compacta*, in the noradrenergic neurons of the *locus coeruleus*, and in sympathetic neurons and in the adrenal medulla (Nagatsu and Ichinose, 1991).

In humans, a single TH gene encodes four main isoforms of TH protein (hTH), generated by alternative splicing of pre-mRNA (Grima et al., 1987). There is no significant difference in the catalytic properties of the four isoforms (Haavik et al., 1991).

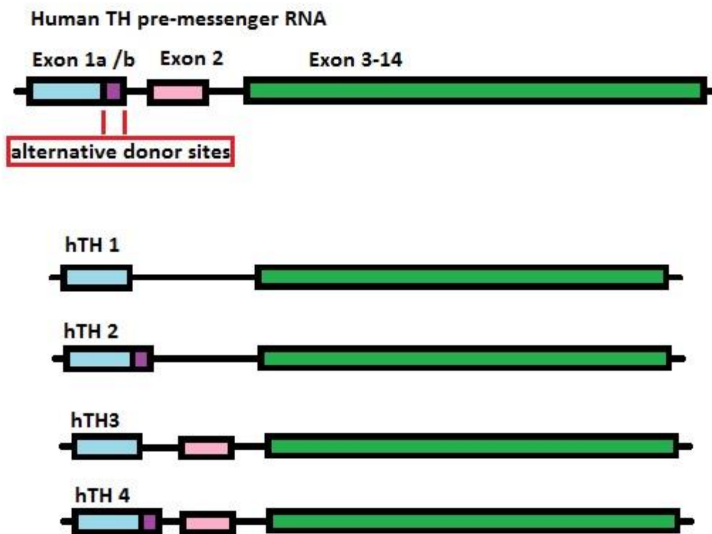

Fig. 4. Alternative splicing of mRNA results from the use of two donor sites in exon 1 and inclusion/exclusion of exon 2 in human TH (hTH). Isoform 1 (hTH1) has no insertion and isoform 4 (hTH4) has the longest insertion of 31 additional amino acid residues.

TH activity is highly regulated, as the catecholamines are essential in a variety of physiological functions. Excess catechol formation is toxic to cells and can lead to protein and DNA damage via oxidation of catecholamines, producing reactive quinones and oxygen radicals (Hastings and Zigmond, 1994; Stokes et al., 1996). Accordingly, TH activity is subject to negative feedback inhibition by catecholamines. The catecholamines coordinate to the ferric iron, leaving TH inactive and preventing the binding of the cofactor BH_4. Human TH (hTH) is phosphorylated by a variety of protein kinases at four Ser/Thr residues (in positions 8, 19, 31 and 40 in hTH1) in the

N-terminal regulatory domain (Andersson et al., 1988; Haycock, 1990; Haycock and Haycock, 1991; Kumer and Vrana, 1996), and phosphorylation of Ser40 by cAMP-dependent protein kinase (PKA) results in a decrease in the K_m for the cofactor BH_4 as well as a reduced affinity for the catecholamine feed-back inhibitors (Le Bourdelles et al., 1991). The detailed molecular mechanisms underlying the catecholamine inhibition are yet to be explicated. However, it has been suggested that there are several different binding modes for the catecholamine inhibition, i.e. first, a high affinity binding of catecholamine to the active site iron causing an irreversible inhibition (Andersson et al., 1988). This produces a significant reduction of the maximum reaction velocity (V_{max}) and disruption of the binding of the cofactor BH_4 (Briggs et al., 2011; Gordon et al., 2008). The high affinity catecholamine binding requires the regulatory domain of TH to be present (Gordon et al., 2009b). This form of catecholamine inhibition can only be relieved by phosphorylation of the serine 40 residue in the regulatory domain, producing a conformational change that allows the catecholamine to dissociate (Almas et al., 1992). A change in TH structure becoming more compact upon binding of catecholamines and more open upon phosphorylation of serine 40 has been demonstrated (Bevilaqua et al., 2001); and is believed to reflect an interaction between the regulatory and catalytic domains. Second, a low affinity binding of catecholamines to TH, producing a reversible inhibition competitive to binding of the cofactor BH_4, but which does not decrease the maximal reaction velocity has been described (Gordon et al., 2008). High and low affinity binding of catecholamines can occur simultaneously, but studies of the interaction between catecholamines and catalytic site residues suggest overlapping sites of high and low affinity binding (Briggs et al., 2011; Gordon et al., 2008). A model of catecholamine binding considering that the size of the active site is not sufficient to bind both catecholamines has been suggested; and a detailed study of the TH dimer has shown that the dimer is probably the core regulatory unit of TH, displaying both high and low affinity catecholamine inhibition, with characteristics indistinguishable from the tetramer, except for cooperativity in catecholamine binding, as expected (Briggs et al., 2014).

TPH 1 and 2 are rate-limiting in the synthesis of the neurotransmitter serotonin and the neurohormone melatonin. There are two isoforms of human TPH encoded by two

different genes, *TPH1* and *TPH2* (Walther and Bader, 2003). The chromosomal locations of the genes differ; *TPH1* is located on chromosome 11, while *TPH2* is on chromosome 12. The nucleotide sequences of the two human enzymes are 70% identical, mainly differing in the regulatory domain. The regulatory domain of TPH 1 contains approximately 88 residues, while TPH 2 contains an additional 46 residues. TPH 1 was the first discovered, and is the most studied enzyme form. TPH 1 is mainly expressed in peripheral tissue, in the enterochromaffin cells in the gut, spleen, thymus, skin and retina; and also in the pineal gland (Cote et al., 2003; Hornung, 2003; Slominski et al., 2003), while TPH 2 is considered to be neuron-specific and expressed in the serotonergic neurons of the raphe nuclei and the myenteric neurons in the gut (Liang et al., 2004; Zill et al., 2004). In the central nervous system (CNS), serotonin acts as a neurotransmitter where it binds to 5-HT receptors; in the pineal gland, it also serves as a precursor for melatonin biosynthesis. In the periphery, serotonin constricts large blood vessels and regulates platelet adhesion; in the intestinal system, serotonin initiates peristaltic and secretory reflexes (Cote et al., 2003; Mawe and Hoffman, 2013; Slominski et al., 2003). TPH activity shows diurnal variation (Liang et al., 2004; Malek et al., 2004). Like the other AAH TPH is subject to regulation by phosphorylation on serine residues in the N-terminal domain.

1.1.5 Enzyme activity essays

The activity of TH has been studied using different assay methods. In 1964, the first report of conversion of L-Tyr to L-DOPA in cell-free preparations of tissue homogenates was published (Nagatsu et al., 1964a). A radiochemical assay using ^{14}C-L-Tyr as a substrate in the conversion reaction, absorbing the resulting catechols on an alumina column was applied to demonstrate a tyrosine hydroxylase activity in the tissue homogenates. Later, an activity assay where ^{3}H-L-Tyr replaced the ^{14}C-L-Tyr as a substrate, measuring the release of tritiated water or the ^{3}H-L-DOPA formed in the catalytic reaction, was developed. This method was argued to be a simpler and less laborious method of activity measurement, as it left the isolation of catecholamines unnecessary (Nagatsu et al., 1964b). Variations of the radiochemical assay also include a method based on the enzymatic decarboxylation of ^{14}C-labelled L-DOPA and

measuring the released $^{14}CO_2$, an assay proposed to be more sensitive than previously described methods (Waymire et al., 1971).

Further interest in developing methods to study TH activity, making labelled substrates and scintillation spectrometers obsolete, resulted in the fluorometric assays (Nagatsu and Yamamoto, 1968; Yamauchi and Fujisawa, 1978). L-DOPA formed in the TH reaction was subjected to the trihydroxyindole derivatization method and the highly fluorescent L-DOPA derivative was isolated from the reaction mixture on columns before fluorometric detection. This method was found to yield comparable results to the radiochemical methods, and used to measure activity in tissues such as brain or adrenal glands accurately, although being less sensitive than the radioassays.

Shiman et al. applied several assay methods to study TH activity in bovine adrenal medulla (Shiman et al., 1971). Comparing to the previously described radiochemical and fluorometric assays, they also presented a colorimetric assay method, taking advantage of L-DOPA's ability to react with nitrous acid in the presence of sodium molybdate to produce a compound that absorbs light with a maximum at 510 nm. They also provide the first description of an oxymetric assay, employing a Gilson oxygen electrode to measure the consumption of oxygen in the TH reaction. Additionally, they measured the substrate dependent oxidation of cofactor by a direct method, recording the increasing absorbance of tetrahydropterins, and indirectly by measuring the absorbance of NADH oxidized in the regeneration of the tetrahydropterins used in the catalytic reaction. All assay methods in their study were compared, and the authors argued that with suitable precautions, both high accuracy and high precision can be obtained. However, these methods have been assessed by others to lack the sufficient sensitivity to be generally useful (Blank and Pike, 1976).

During 1970-1980, new assay methods applying high performance liquid chromatography (HPLC) were developed (Blank and Pike, 1976). By passing tissue homogenates and other reaction mixtures through a HPLC column, separation of components in the reaction mixture may be achieved, and L-DOPA formed in the TH reaction can be quantified by electrochemical (Blank and Pike, 1976; Naoi et al., 1988;

Philipp, 1987) and fluorometric detection (Bailey and Ayling, 1980; Bailey et al., 1989; Haavik and Flatmark, 1980). Common advantages for the HPLC based assay methods are their high sensitivity, reliability and precision, less expensive reagents, and being less laborious and time consuming compared to radiochemical methods.

	Detection method	**Sensitivity - Lower limit of detection**	**Signal-to-noise ratio**	**Reference**
Radiochemical assay	Scintillation counting of			
	^{14}C-L-DOPA	~ 5 pmol	n.s.	(Nagatsu et al., 1964a)
	$^{14}CO_2$	5 pmol	n.s.	(Waymire et al., 1971)
	^{3}H-L-DOPA	~ 5 pmol	n.s.	(Nagatsu et al., 1964c)
	$^{3}H_2O$	~ 5 pmol	n.s.	(Nagatsu et al., 1964b)
Fluorometric assay	Direct measurement of			
	L-DOPA	100 pmol	2	(Nagatsu and Yamamoto, 1968; Nagatsu et al., 1979b)
	L-DOPA	100 pmol	n.s.	(Yamauchi and Fujisawa, 1978)
	tetrahydropterin	n.s.	n.s.	(Shiman et al., 1971)
	(Indirect) tetrahydropterin regeneration	n.s.	n.s.	(Shiman et al., 1971)
Oxygraphic assay	Direct measurement of oxygen consumption			
	Gilson electrode	n.s.	n.s.	(Shiman et al., 1971)
HPLC based assay				
	Electrochemical-voltametric	0,2 pmol	3	(Blank and Pike, 1976)
	Electrochemical-voltametric	2 pmol	3	(Philipp, 1987)
	Electrochemical-voltametric	5 pmol		(Nagatsu et al., 1979a)
	Electrochemical-coulometric	0,25 pmol	5	(Naoi et al., 1988)
	Fluorometric	5 pmol	3	(Haavik and Flatmark, 1980)
	Fluorometric	1 pmol	2	(Bailey et al., 1989)

Table 1. Summary of the assay methods developed for the tyrosine hydroxylase reaction. Sensitivity is reported according to the original articles published. *N.s.= not specified*

1.1.6 Regulation of AAH activity with emphasis on their phosphorylation and interaction with 14-3-3 proteins

Phosphorylation of serine residues in the regulatory amino-terminal domain is a common mode of short-term regulation of the AAHs (Daubner et al., 2011). All four AAHs are phosphorylated by cAMP-dependent protein kinase (PKA) and Ca^{2+} /calmodulin dependent protein kinase (CaMKII) (Fitzpatrick, 2000; Winge et al., 2008); upon phosphorylation the enzymes are activated and/or stabilized; and phosphorylation of TH and TPH facilitates binding of 14-3-3 proteins (Ichimura et al., 1987; Kleppe et al., 2001). Other protein kinases can phosphorylate AAHs; however, only PKA and CaMKII are mentioned here as they are the most studied kinases involved in the regulation of the AAHs.

Phosphorylation of PAH at Ser16 increases the basal activity of PAH; a localized conformational change in the structure of the enzyme leading to a more “open” active site (Miranda et al., 2002; Miranda et al., 2004), thus less phenylalanine is required to activate the hydroxylation reaction (Doskeland et al., 1996). In addition, phosphorylation seems to decrease the rate of proteolysis of PAH (Miranda et al., 2004).

TPH 1 is phosphorylated on Ser58 by PKA (Johansen et al., 1996; Vrana et al., 1994) leading to activation of the enzyme; while TPH 2 phosphorylation occurs on Ser19 by CaMKII and PKA (Kuhn et al., 2007; Winge et al., 2008) and on Ser104 by PKA, corresponding to Ser58 in TPH 1 (Winge et al., 2008).

Compared to PAH and TPH 1 and 2; phosphorylation and interaction with 14-3-3 proteins for TH have been extensively studied. TH can be phosphorylated on four serine residues; Ser8 (Thr8 in some species, including human TH), Ser19, Ser31 and Ser40. Several kinases are involved in phosphorylating TH, and show specificity in the serine residue they phosphorylate (Dunkley et al., 2004). Increased TH activity is seen primary upon phosphorylation on Ser40; which both increases the affinity for the cofactor BH_4 and contributes to alleviate the catecholamine feedback inhibition (Almas et al., 1992; Daubner et al., 1992; Haavik et al., 1990; Le Bourdelles et al., 1991). On

the other hand, phospho-Ser40 TH is more susceptible to thermal denaturation *in vitro*, possibly reflecting a conformational change, leading to a more unstable enzyme (Gahn and Roskoski, 1995; Lazar et al., 1981; Ramsey and Fitzpatrick, 2000). Phosphorylation of Ser31 also activates TH by increasing the affinity of TH for BH_4 (Halloran and Vulliet, 1994; Haycock et al., 1992; Sutherland et al., 1993). However, no evidence for relieving of the catecholamine inhibition upon Ser31 phosphorylation was found (Haycock et al., 1992; Salvatore et al., 2001; Wu et al., 1992). *In situ* studies indicate that Ser31 phosphorylation occurs later than Ser40 and Ser19 phosphorylation in response to depolarization stimuli (Thomas et al., 1997; Waymire et al., 1988). Evidence for differences in phosphorylation on Ser31 between the different isoforms of hTH has been found, where the Ser35 in hTH2 (corresponding to Ser31 in hTH1) was not phosphorylated in response to stimuli in a cell culture when the same conditions lead to phosphorylation of Ser31 in hTH1. Further, the phosphorylation of Ser31-hTH1 increased the phosphorylation of Ser40, an effect that was absent in the hTH2 (Gordon et al., 2009a). Compartmental differences of TH phosphorylated on Ser31 was shown in a study comparing TH phosphorylation in rat striatum, *substantia nigra*, ventral tegmental area and *nucleus accumbens* (Salvatore and Pruett, 2012). These findings propose a difference in regulation between the TH isoforms.

Phosphorylation of Ser19 in TH does not activate the enzyme directly either *in vitro* or *in situ* (Sutherland et al., 1993; Toska et al., 2002). There are, however, evidence for important regulatory consequences for phosphorylation of this residue in TH. First, a hierarchical regulation of phosphorylation of TH has been suggested, where Ser19 phosphorylation leads to a conformational change in TH increasing the access to phosphorylation of Ser40 (Bevilaqua et al., 2001; Toska et al., 2002). Second, phosphorylation of Ser19 is essential in interaction of TH with stabilizing/activating proteins, e.g. 14-3-3 proteins, as discussed below.

No evidence for an effect on TH activity or catecholamine production have been found for phosphorylation of TH Ser/Thr8 (Dunkley et al., 2004).

The 14-3-3 proteins are regulatory molecules present in all eukaryotic species, in mammals 7 different genes codes for different isoforms. The 14-3-3 isoforms β, γ, ε, η, τ and ζ are expressed abundantly in brain, and are shown to regulate TH and TPH activity (Ichimura et al., 1988).While all four AAHs have similar ACT regulatory domains, TH and TPH 2 have a long N-terminal extension (residues 1-43 in hTH1) that contain the Ser19 residue facilitating interaction with 14-3-3 proteins (Murphy et al., 2008; Skjevik et al., 2014; Winge et al., 2007). Phosphorylation of TH Ser19 is shown to initiate binding of 14-3-3 proteins (Daubner et al., 2011; Halskau et al., 2009; Kleppe et al., 2014), and there is also evidence for binding to 14-3-3 proteins for phospho-Ser40-TH and double phosphorylated Ser19-Ser40-TH (Hritz et al., 2014; Kleppe et al., 2001). The 14-3-3 proteins can be both monomers and dimers, and form homo- or heterodimers. In dopaminergic cells, phospho-Ser19-TH interacts with multiple 14-3-3 dimer isoforms, resulting in activation of TH and inhibition of its dephosphorylation (Ghorbani et al., 2016). Structural studies of the N-terminal peptide (1-43) of TH reveals a conformational change upon binding of 14-3-3γ by phospho-TH-Ser19; giving a more extended structure compared to non-phosphorylated TH (Skjevik et al., 2014).

1.2 AAHs in human disease

1.2.1 Phenylketonuria as a model disease for studying the effects of mutations in aromatic amino acid hydroxylases

Classical phenylketonuria (PKU), or Følling's disease, is an inborn defect in the ability to metabolize the amino acid phenylalanine (Scriver, 2007; van Spronsen, 2010), causing increased levels of phenylalanine in the blood. The disease was discovered in 1934, when dr. Følling found phenylpyruvic acid in the urine of a pair of siblings with mental retardation (Følling, 1934). PKU can be categorized by severity based on the phenylalanine levels at diagnosis and dietary tolerance to phenylalanine; classical PKU (blood levels of L-Phe >1200 µmol/L), mild PKU (600–1200 µmol/L) and mild hyperphenylalaninemia (120–600 µmol Phe/L) (Blau et al., 2010). Atypical PKU can

also occur due to congenital deficiency of BH_4 synthesis (Shintaku, 2002). The symptoms of PKU can vary from mild to severe, and without treatment the child can develop intellectual disability, and in severe cases seizures, delayed development, behavioral problems and psychiatric disorders like depression and anxiety (Brumm et al., 2010; Pey et al., 2007; Scriver, 2007). In Norway an average of 3-5 children are born with this genetic defect every year. Since 1978, the Norwegian national post-natal screening program has included screening for PKU. With early treatment with a phenylalanine restricted diet and supplementation with essential amino acids, development of disability can be avoided (Bickel et al., 1953; Enns et al., 2010; Smith and Knowles, 2000).

To date, more than 950 different mutations in PAH are reported (www.biopku.org/pah, accessed March 2016). The majority of the mutations are missense mutations (approx. 60 %), the remaining mutations are small deletions (13 %), splice mutations (11%), putative silent mutations (7 %), stop/nonsense mutations (5 %), small insertions (1 %), and large deletions (up to 3%) (Kozak et al., 2006; Scriver et al., 2003). In extensive population studies associating genotype with biochemical phenotype in the patients (Desviat et al., 1997; Eisensmith and Woo, 1995; Guldberg et al., 1998) and in which the severity of most mutations can be predicted, there are notable inconsistencies for a few missense mutations, in the sense that they are associated with variable phenotypes in patients with identical genotypes. Several of the mutated variants of PAH found in PKU patients have been characterized *in vitro*, and the activity, kinetic properties and stability of mutated enzyme have been studied. The main molecular mechanism of enzyme dysfunction is considered to be instability and misfolding of the enzyme, leading to degradation and/or aggregation of the protein (Gersting et al., 2008; Pey et al., 2007; Wettstein et al., 2015).

The mechanisms of the cognitive impairment in PKU are not fully solved, but a current hypothesis involves the role of hyperphenylalaninemia on the function of the other aromatic amino acid hydroxylases, TH and TPH1/TPH2. Furthermore, an affection of protein synthesis in the brain has been reported, leading among other to dysfunction in myelin formation by glia cells (oligodendrocytes) (de Groot et al., 2010). Recently, an

amyloidosis-like pathophysiology has also been suggested for PKU, based on evidence for formation of neurotoxic fibrils of phenylalanine (Adler-Abramovich et al., 2012). The transport of large neutral amino acids (LNAA) (phenylalanine, tyrosine, tryptophan, leucine, isoleucine, valine, histidine, methionine and threonine) across the blood-brain barrier is mainly carried out by the LAT-1-transporter. The LAT-1 transporter binds the LNAA with different affinities, and is also a counter-transporter, e.g. for each LNAA transported into the brain, another is transported out (Zielke et al., 2002). This is a competitive process, and hyperphenylalaninemia leads to a higher transport of phenylalanine into the brain, reducing transport of the other LNAAs as a consequence (van Spronsen et al., 2010). L-Phe is also a competitive substrate for TH (Fukami et al., 1990). Low concentrations of tyrosine and tryptophan in the brain, the substrates of the rate-limiting steps in the production of the neurotransmitters dopamine, noradrenaline and serotonin, respectively, can lead to dysfunction in neuronal signaling (de Groot et al., 2010). The protein production in the brain is also compromised, with potentially extensive consequences (de Groot et al., 2010; Moller et al., 1998; Weglage et al., 1998).

1.2.2 Tyrosine hydroxylase deficiency

Congenital tyrosine hydroxylase deficiency (THD) is found in DOPA responsive dystonia and some related neurological syndromes with predominantly motor symptoms. The clinical manifestations of THD are variable, with different levels of severity, age of onset and symptom profiles. THD patients are clinically categorized as type A or B; type A representing the group of patients with mild parkinsonian symptoms and type B, those with a more severe early onset lethal disease. THD appears to have an exclusively autosomal recessive inheritance. The approx. 70 patients who have been described so far are either homozygous or compound heterozygous for mutations in the exons or the promoter region of TH gene. From 1995-2012, ~ 50 different disease related missense mutations and 4 nonsense mutations in the TH gene have been reported. Additionally, 3 mutations in the promoter region of the TH gene have been found in THD (Willemsen et al., 2010).

Recently, two different knock-in mouse models of tyrosine hydroxylase deficiency have been studied (Korner et al., 2015; Rose et al., 2015). The mouse models carry the R203H and Q382K mouse *Th* mutations, respectively, corresponding to the human TH4 R233H and Q412K mutations. Both knock-in mice homozygous for mutations displayed dystonic movements with diurnal fluctuations and hypokinesia; the Q382K knock-in mice had significant improvement in the symptoms upon L-DOPA or trihexyphenidyl supplementation, while the R203H mice did not respond to supplement treatment. The mice seem to represent good models for each of the sub- types of THD; the first carrying the R203H mutation displaying features of the type B THD and the second, Q382K, of type A, in accordance with the phenotype association of each mutation in human THD (Willemsen et al., 2010).

1.2.3 Parkinson's disease/syndrome

Parkinson disease (PD) is a chronic and progressive neurological disease associated with a loss of dopaminergic neurons. In most cases the disease is sporadic, but genetically inherited cases also exist (Ross and Farrer, 2005). One of the major pathological features of PD is the presence of protein aggregates that localize in neuronal cytoplasm as Lewy bodies, mainly composed of α-synuclein (α-syn) and ubiquitin. Immunohistochemical studies of the Lewy bodies of PD in post mortem brains have also provided evidence for catechol and TH inclusions (Goldstein et al., 2011). The selective degeneration of dopaminergic neurons suggests that dopamine itself may contribute to the neurodegenerative process in PD. Degenerating neurons in the *substantia nigra* down-regulate the expression of dopamine transporter and TH; a paradox in the dopamine depleted neuron that is not fully understood (Obeso et al., 2008). In the TH reaction, reactive oxygen species can be formed if the geometry of the active site is altered or alternative substrates are available (Haavik and Toska, 1998). Reactive oxygen species can damage proteins and DNA, leading to cell damage (Perfeito et al., 2012). L-DOPA has been shown to be a substrate for TH, with comparable V_{max} and K_m values as for tyrosine (Haavik, 1997). Oxidation of L-DOPA by TH is thought to mediate formation of neuromelanin and possibly to be involved in

the L-DOPA toxicity. Thus, a direct pathogenic role of TH in the neurodegenerative process of PD has been suggested (Haavik and Toska, 1998).

1.2.4 A possible role of TH in hypoxia induced disease

At higher altitudes the barometric pressure decreases, leading to lower oxygen concentration in the air. Before acclimatization occurs, hypoxia causes activation of the sympathetic nervous system and thereby increased respiration, blood pressure and heart rate. Altitude sickness is a pathological effect of high altitude in humans, presenting with unspecific symptoms such as headaches, nausea, fatigue, dizziness and sleep disturbances (Joseph and Pequignot, 2009). Acute altitude sickness can progress to high altitude cerebral edema or pulmonary edema which is fatal; however, in most cases the acclimatization process relieves the symptoms (Joseph and Pequignot, 2009; Wilson et al., 2009). Studies in humans have shown a temporary reduction in catecholamine synthesis during hypoxia (Leuenberger et al., 1991; Sevre et al., 2001). It has also been demonstrated that the concentration of plasma catecholamines correlates well with the peripheral oxygen saturation in a study of participants of a climbing expedition (Rostrup, 1998). Additionally, there have been several reports of normobaric hypoxia reducing the *in vivo* tyrosine hydroxylation in rat brain (Brown et al., 1974; Davis and Carlsson, 1973; Davis, 1975). Reduced brain noradrenaline after hypoxia has also been found (Hayashi et al., 1990). Reported TH K_m-values for O_2 vary from 3-24 μM (Fisher and Kaufman, 1972; Fitzpatrick, 1991; Ikeda et al., 1966; Katz, 1980; Numata et al., 1977). Although the O_2 concentration in brain, kidney and liver tissue of living animals is uncertain (Feinsilver et al., 1987; Rolett et al., 2000), it is likely that at sea level the tissue partial pressures of oxygen may get close to the K_m-levels suggested for rat TH and be rate limiting. Thus, at high altitude, the partial oxygen tension in peripheral tissue of humans most probably will reach levels in the range of the K_m-values of rat TH, especially in subjects not fully acclimatized.

Chronic intermittent hypoxia, as seen in obstructive or central sleep apnea syndrome (OSAS, CSAS), is associated with hypertension, metabolic abnormalities such as hyperglycemia and hypercholesterolemia and increased risk of cardiovascular disease (Passali et al., 2015; Toraldo et al., 2015). Hypoxia is sensed by the carotid bodies

which evokes increased sympathetic nerve activity (Prabhakar et al., 2015), and increased activity of the adrenal medulla catecholamine production has been demonstrated (Peng et al., 2014). A role of TH in sleep apnea syndrome has been suggested, and studies of intermittent hypoxia (IH) on PC12 cells have demonstrated involvement of short term regulatory mechanisms, e.g. serine phosphorylation, of the TH enzyme in response to IH (Kumar et al., 2003). Hypoxia can also lead to increased *TH* gene expression via the actions of Hypoxia-inducible factor-1α, thus regulating TH activity in cells on a long term scale (Lim et al., 2015).

1.2.5 Human tryptophan hydroxylase associated disease

All four of the AAHs have been proposed as candidate genes in psychiatric disorders. For *TH* and *PAH*, the reports of association so far have been inconsistent (De Luca et al., 2008; Jacobsen et al., 2015; Meloni et al., 2002; Sobell et al., 1993; Wilcox et al., 2002). Association with both *TPH 1* and *2* for different psychiatric disorders like depression, suicidal behavior, attention deficit hyperactivity disorder (ADHD) and anxiety; are reported in several studies (Bach-Mizrachi et al., 2006; Boldrini et al., 2005; Cichon et al., 2008; McKinney et al., 2008). Mutated forms of TPH2, associated with reduced serotonin synthesis in the brain, P449R, major depressive disorder, R441H, bipolar affective disorder, P209S and ADHA, R303W have been characterized (Winge et al., 2007). All of the variants of TPH2 show reduced solubility and thermal stability compared to wild-type (wt)-TPH2. The mutants R449H and R303W also have reduced enzymatic activity compared to wt-TPH2, in contrast to P209S and P449R that have similar specific activity as wt-TPH2.

2 Methods

2.1 Protein expression, purification and mutagenesis

For the study of THD associated variants of TH, mutations were introduced into the wt-hTH1 cDNA on the pET3a-hTH1 vector (Haavik et al., 1991) by PCR-based mutagenesis using the QuikChange mutagenesis kit (Stratagene, La Jolla, CA, USA). Introduction of the correct mutation and exclusion of other mutations was verified by Sanger sequencing of the whole coding region. Recombinant human TH, isoform 1 (hTH1) and the mutant hTH1 were expressed in BL(21)D3pLysS *Escherichia coli* (Invitrogen™). The bacteria were grown at 37℃ in LB medium containing 50 µg/ml of ampicillin and 34 µg/ml of chloramphenicol. The expression of T7 polymerase was induced at $OD_{600\ nm}$ = 0.8 by addition of 1 mM isopropyl 6-D-thiogalactopyranoside. The temperature was then decreased to 25℃, and the bacteria were harvested after 6 hours incubation. The bacteria pellets were kept at -20℃ until purification.

Bacteria (from 1 L of culture) were diluted in 20 mM Tris/HCl, containing *5%* mass/vol. sucrose, 1 mM dithiothreitol (DTT), 1 mM EDTA, 0.1 mM phenylmethylsulfonyl fluoride and 1 tbl. *Complete Protease inhibitor cocktail, EDTA-free* (Roche, Mannheim, Germany) /25 mL, and disrupted by passage through a French press (type FA-073 from SLM Instruments, Urbana IL) at 69 MPa. The lysate was centrifuged at 12 000 *g* for 20 min., and the supernatant was purified by heparin Sepharose chromatography. The purified enzymes were concentrated and stored in liquid nitrogen until used.

2.2 TH enzyme activity assay

In addition to the required substrates and cofactor for the aromatic amino acid hydroxylation to occur; the aromatic amino acid substrate, molecular oxygen, ferrous iron and a reduced pterin cofactor; the reaction mixture in our studies contains catalase and DTT. Catalase and DTT are beneficial for protecting the enzyme activity as scavenger of H_2O_2 and a reductant for the cofactor, respectively (Cash, 1998). The natural cofactor, BH_4, was used in the studies presented.

2.3 The oxygraphic method of studying aromatic amino acid hydroxylases

We applied the Oroboros 2K oxygraph (Oroboros Instruments, Innsbruck; and Paar, Graz, Austria) (Fig. 5A). This is a digital oxygraph with optimized reaction chamber geometry, low background noise levels, and possibilities for addition and removal of samples during analysis. Initial oxygen concentrations were varied by adding pure argon (99.996%) into the gas phase of the partially opened oxygraph chamber. The chamber was closed for recording of chemical oxygen consumption before the reaction was initiated by addition of enzyme. The oxygen flux was analyzed using the DatLab software (Oroboros Instruments, Innsbruck), which includes online calculation of the time derivative of oxygen concentrations and correction for instrumental background oxygen flux.

A.

B.

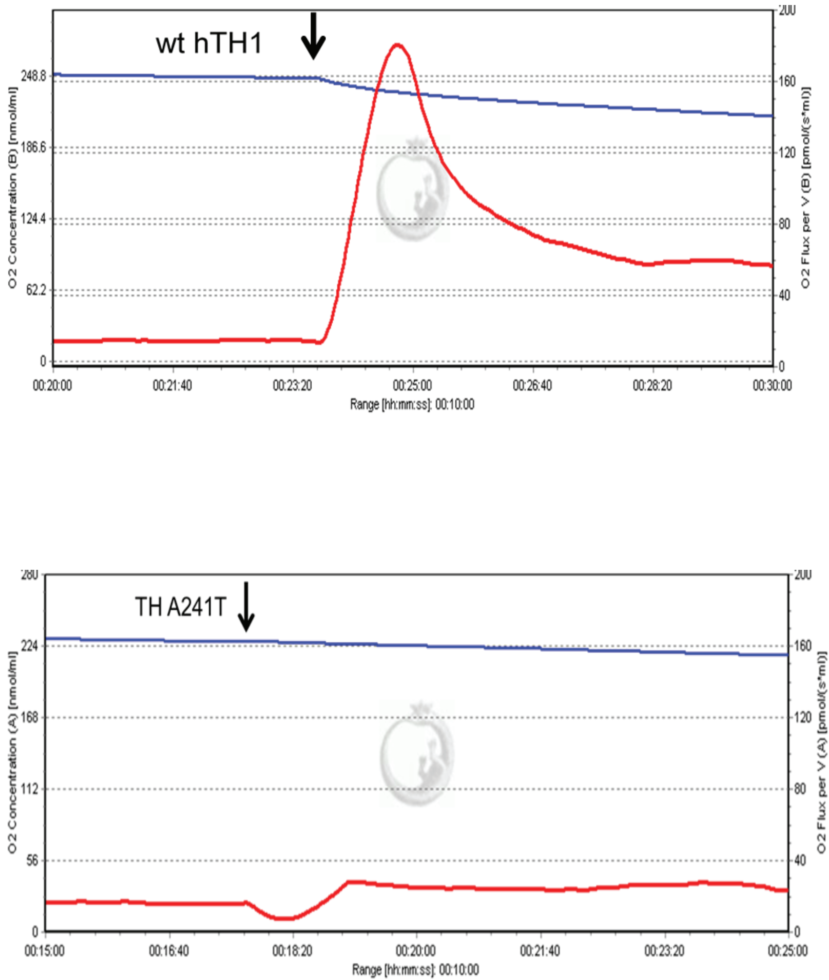

Fig. 5. Oxygraphy

A. The Oroboros Oxygraph 2K
B. Real time oxygraph recording of oxygen consumption of WT and mutant TH. An oxygraph recording of the oxygen consumption during tyrosine hydroxylation is represented by the blue trace, left axis. The reaction is started by hTH addition, indicated by an arrow. The reaction velocity v_{O_2} (nmol·min^{-1}·mg^{-1}) is given by the red trace, right axis. After addition of 10 μg TH, the oxygen flux was measured directly using an oxygen electrode.

3 Aims of the present study

To increase our understanding of the activity and regulation of the AAHs, as well as their implication in disease due to mutations that affect their function and stability. The work was organized in subprojects to answer specific aims 1-4:

1. To develop improved methods of studying aromatic amino acid hydroxylases (AAHs), mainly focusing on their dependence of oxygen.

2. To investigate the role of oxygen in the AAH reactions, with an emphasis on TH.

3. To characterize the effect of mutations in AAH, mainly PAH and TH, on their enzyme function and stability.

4. Investigate the effects of missense *TH* mutations reported in patients with TH deficiency and perform genotype-phenotype comparisons in these patients.

4 Summary of articles

4.1 Article 1; An oxygraphic method for determining kinetic properties and catalytic mechanism of aromatic amino acid hydroxylases

The first part of this project was focused on developing an oxygraphic method for determining kinetic properties and catalytic mechanism of aromatic amino acid hydroxylases. We applied the *Oroboros Oxygraph 2k* for continuously measuring the oxygen consumption in the hydroxylase reaction. Measurement of other substrates and products in the reaction was possible by removing aliquots of reaction mixture during the experiment and using HPLC for analysis.

We were able to demonstrate the oxygraphic method as an uncomplicated and versatile tool for studying the AAHs, with fairly high sensitivity and time resolution. At a signal-to-noise ratio of 3, we could measure enzyme activity down to ~ 1.8 nmol dioxygen/min/ml and recording of the oxygen concentration in the reaction mixture every 200 msec. Determination of kinetic properties of both wild-type and mutant protein and different substrates and cofactors was possible. A stable reaction stoichiometry of 1:1 was obtained between the amount of oxygen consumed and the tyrosine formation when the natural cofactor (6R)-tetrahydrobiopterin was added as electron donor in the phenylalanine hydroxylase (PAH) reaction. In comparison, low and variable coupling efficiency values between oxygen consumption and tyrosine formation were found using the parent unsubstituted tetrahydropterin. Furthermore, we studied the phenylketonuria-associated PAH mutant R158Q and found that the reaction had a coupling efficiency of about 80 % compared to the wild-type enzyme under similar conditions. In this reaction the amount of H_2O_2 produced in the reaction catalyzed by R158Q PAH was about four times higher than the amount produced by the wild-type PAH, demonstrating a possible pathogenetic mechanism of the mutant enzyme.

4.2 Article 2; Oxygen dependence of tyrosine hydroxylase

In this part of the project we focused on the effects of oxygen concentration on the TH reaction. The effect of dioxygen on TH activity was studied, measuring the formation of DOPA from tyrosine, ^3H_2O from 3,5-^{3}H-tyrosine, or by direct oxygraphic determination of oxygen consumption. During the initial 1–2 min of the reactions, we observed a phase of high enzyme activity, followed by a decline in activity. Apparent K_m-values of 29–45 μM for dioxygen were determined for all human TH isoforms in the initial high activity phase, i.e. 2–40 times higher than previously reported for TH isolated from animal tissues. After 8 min incubation, the K_m (O_2)-values had declined to an average of 20 ± 4 μM. The lower activity phase is possibly related to a turnover dependent substoichiometric oxidation of enzyme bound Fe(II) to the inactive Fe(III) state. We concluded that TH activity may be severely limited by oxygen availability even at moderate hypoxic conditions, and that the enzyme is rapidly and turnover dependent inactivated at the experimental conditions commonly employed to measure *in vitro* activities.

4.3 Article 3; Functional studies of tyrosine hydroxylase missense variants reveal distinct patterns of molecular defects in Dopa-responsive dystonia

The reported clinical manifestations of THD are highly variable, with different levels of severity, age of onset and symptom profile. We collected clinical and biochemical data for all variants described in the literature at the start of the project. Thereafter, we also generated mutant forms of TH that had not previously been characterized biochemically in the literature, using site-directed mutagenesis and isoform 1 of human TH (TH1) as template. All TH mutants were expressed in *E. coli* (BL21(D3) pLysS) and purified on a heparin-Sepharose column. We compared the *in vitro* solubility, thermal stability, and kinetic properties of the TH variants to determine the cause(s) of their impaired enzyme activity. Interestingly, some TH variants had specific kinetic anomalies; and phenylalanine hydroxylase, and L-DOPA oxidase activities were measured for variants that showed signs of altered substrate binding. We found shifted

substrate specificity from tyrosine to phenylalanine and L-DOPA for the TH mutants p.Arg233His, p.Gly247Ser, and p.Phe375Leu, whereas p.Cys359Phe had an impaired activity toward these substrates. The great heterogeneity in solubility, stability and enzymatic activity of the mutated forms of TH, indicates that different pathogenic mechanisms may be involved in the neurological syndromes related to THD.

5 General discussion

5.1 Activity assays of tyrosine hydroxylase

As described in the introduction, several assay methods for studying the activity of TH have been developed. Prior to the availability of recombinant enzyme, challenges concerning low enzyme availability and low enzyme activity in tissue homogenates caused the activity assays to mainly rely on spectrophotometric or other more sensitive procedures, using ^{3}H- or ^{14}C-labeled substrates or measuring product formation by HPLC with electrochemical or fluorometric detection (Haavik and Flatmark, 1980; Hooper et al., 1997; Kappock and Caradonna, 1996; Vrana et al., 1993). An oxygraphic method using a Gilson electrode to measure the oxygen consumption in the enzyme reaction has been published earlier (Shiman et al., 1971). However, this method had a low time resolution (5-8 min. between recordings) and has been criticized for low sensitivity by other researchers (Blank and Pike, 1976). As the oxygen consumption in enzymatic reactions of AAHs can provide additional mechanistic information, and is relevant in pathogenic mechanisms, we developed an oxygrapic assay (Article 1) using the *Oroboros Oxygraph 2k*, originally manufactured for monitoring oxygen consumption in mitochondria and cell preparations (Gnaiger, 2001; Hutter et al., 2004). Compared to the radiochemical and HPLC based assay methods, our oxygrapic assay has a relatively low sensitivity and throughput. However, it has given mechanistic and kinetic information previously unknown, such as new insight into the initial phase of the enzymatic reaction of the AAHs. Furthermore, combining measurement of the oxygen consumption and product formed in the enzyme reaction is necessary for uncovering "uncoupled" catalysis, particularly relevant for studying disease associated mutations.

5.2 Oxygen dependence of TH and production of ROS

Availability of molecular oxygen is essential for respiration in all animal tissues and many lower organisms. Similarly, oxygen is a substrate for many biosynthetic reactions. In our study of the oxygen dependence of TH (Article 2), we found that the enzyme is rapidly inactivated at the experimental conditions usually employed to measure *in vitro* activities. We demonstrated high initial reaction rates in the hTH reaction, with initial K_m'-values indicating that the enzymatic activity may be severely limited by oxygen availability under such conditions. Moreover, we have described a turnover dependent inactivation of the enzyme using several independent assay procedures. The transition from the high to the low-activity phase could either be due to a slow conformational change or a modification of the enzyme, e.g. by oxidation of the active site iron, with accumulation of an inactive Fe(III) state (Chow et al., 2009; Frantom et al., 2006), or by oxidation of redox sensitive amino acid residues. Evidence for both types of reactions has previously been presented for TH and other pterin dependent hydroxylases (Wallick et al. 1984; Ramsey et al. 1996; Kuhn et al. 1999). During catalytic turnover, the regeneration of enzyme bound Fe(II) may become kinetically limiting. Thus, Frantom et al. have calculated the half-life of reduction of Fe(III)TH by BH4 to be 3-5 s under physiological conditions (Frantom et al., 2006). We studied the effects of the Fe(III) chelator desferrioxamine on the TH reaction, observing a time dependent inhibition of the enzyme similar to the observations of Ramsey et al. (Ramsey et al., 1996) using dihydroxynaphthalene on the rate of inactivation of rat TH. These findings indicate that a substantial amount of the active site Fe(II) is oxidized within the first 1-2 minutes of the enzyme reaction. As the kinetic burst cannot be overcome by increasing the exogenous concentrations of Fe(II), we suggest that it is the reduction of active site Fe(III), rather than incorporation of "new" Fe(II) that becomes rate limiting during the steady state TH reaction.

As described in the introduction, the enzymatic reaction of the AAHs is dependent on the presence of a ferrous iron, correct sequence of binding and positioning of cofactor and substrates in the catalytic site. Of the THD associated mutations reported to date,

the C359F mutation, as one of 6 conserved cysteines in TH, is the mutation most likely to interfere directly in the catalytic site of TH. In our study (Article 3), we found that the C359F-TH mutant has a severely reduced activity compared to wt-TH (~10 %) and significantly altered affinities for both tyrosine and the cofactor BH_4. Other catalytic domain variants of mutated TH also had altered properties; F375L-TH revealed a low specific activity (~ 10 % of compared to wt-TH) and a very low affinity towards tyrosine. Further investigation showed that the F375L-TH mutant had identical catalytic properties for phenylalanine hydroxylation as for tyrosine hydroxylation. The A376V-TH mutant had significantly reduced solubility compared to wt-TH, but had comparable substrate affinity and substrate inhibition constants. L387M-TH has solubility and activity comparable to wt-TH, but severely decreased thermal stability. The mutations I394T-TH and T399M-TH are also close to the active site, I394M-TH has severely reduced solubility and no measureable activity. T399M-TH has severely reduced activity and thermal stability. Active site mutations in the AAHs may interfere with normal reaction mechanism, producing toxic by-products. Excess oxygen consumption in the enzyme reaction can lead to formation of reactive oxygen species (ROS) (Haavik et al., 1997). We found no evidence for excess oxygen consumption in the mutant TH forms in this study. However, formation of ROS can be a pathogenic mechanism *in vivo*, as proposed for PD (Haavik and Toska, 1998; Nakashima et al., 2013).

5.3 Pathophysiology of AAH and comparison to findings in this study

Understanding the molecular events causing human disease is one of the great challenges in the post-genomic era. Generally, it is difficult to obtain strict genotype-phenotype correlations, even for well-defined monogenic metabolic diseases (Weatherall, 2000). This can partly be explained by the existence of compensatory physiological mechanisms and that many genes/proteins interact to create the genetic background through which the genotype has to penetrate, thus forming the observed phenotype. An example of this is found in PKU, where a proposed protective mechanism for hyperphenylalaninemia is described in patients with a polymorphism

in a transaminase that increases the clearance of phenylalanine from the bloodstream (Treacy et al., 1996).

For THD, the establishment of a clear correlation of genotype and phenotype is further complicated by the small number of patients reported, and the fact that a majority of the patients are compound heterozygous for mutations in the TH gene. Moreover, homozygosity of the p.Arg233His mutation has been reported in six type A and three type B patients. The p.Leu236Pro mutation occurred homozygously in one type A and two type B patients. However, all patients with at least one promoter mutation had the type A THD, indicating that some TH protein was produced, despite the presence of the promoter mutation. In THD patients born of consanguine parents, there is also an increased risk of other autosomal recessive traits/diseases that can complicate the clinical symptoms. There has nevertheless been reported a correlation between some biochemical phenotypic markers, as the CSF HVA content in the patients and the severity of their THD, based in their A and B-type classification (Willemsen et al., 2010). A strong correlation between the activity of the mutants and CSF HVA content was found in our study. Thus, we also obtained a relationship between the remaining activity and the THD subtype (A and B) of the patients (Article 3). In the recently published studies of THD mouse models, the phenotype is homogeneous in the homozygous mice, probably reflecting the genetic background homogeneity of the inbred strain of mice used for genetic studies. Human patients, even homozygous for mutations in TH, have a higher phenotypic variability (Willemsen et al., 2010), suggesting a possibility for THD as a complex trait autosomal recessive disorder.

In their study of the knock-in mouse model of THD type A carrying the homozygous mouse *Th* p.Q382K, Rose et al. (Rose et al., 2015) demonstrated reduced TH activity *in vivo* corresponding to the activity measured for the purified recombinant hTH1 p.Q381K *in vitro* of ~ 15 % compared to wild type enzyme in brain regions containing mainly cell soma. Interestingly, in the axon terminal regions the TH activity was severely reduced (~ 1 % of wild type) possibly reflecting the reduced content of TH enzyme in the axon terminals.

Korner et al. have studied the THD type B associated mouse *Th* p.R203H mutation (corresponding to human TH1-p.R202H) knock-in mouse; a model of the most frequently occurring mutation found in human THD. The mice display a phenotype of severe dystonia with diurnal fluctuations, growth retardation despite normal calorie intake and respiratory exchange ratio, and no relief of symptoms upon administration of L-DOPA. A distinct absence of TH protein was observed in the striatum of this mouse model, as for the former.

To date, no animal model of THD carrying TH mutations biochemically characterized in our study has been investigated, although both the p.R202H and the p.Q381K mutations were reviewed in Article 3. However, the present publications of THD knock-in mice models illustrates the value of knowledge of the biochemical characterization of mutated enzyme variants to explain *in vivo* effects and provide insights to focus research questions and areas of examination. The reduced enzyme stability shown in *in vitro* studies of the hTH1-p.Q381K (Knappskog et al., 1995) and hTH1-p.R202H (Calvo et al., 2010; Korner et al., 2015) may indicate an explanation for the reduced transport of protein from the cell soma to the axon terminals, thus providing biochemical insight to the mislocalization of TH. Furthermore, *in vivo* examination of the consequences of altered substrate affinity and shift in substrate specificity of some of the mutated TH variants found in our study could elucidate disease processes still unknown and provide alternatives for specific therapeutic strategies. Dopamine deficiency, caused by degeneration of catecholaminergic neurons in PD or by primary THD, is usually treated by administration of L-DOPA or dopamine agonists. High levels of L-DOPA can even be neurotoxic and be oxidized to reactive intermediates, including quinones, semiquinones, and hydrogen peroxide (Kostrzewa et al., 2002). For the mutants of TH p.Arg233His, p.Gly247Ser, p.Phe375Leu and p.Gly414Arg, we observed a relatively increased efficacy of L-DOPA as substrate. Further investigation of these variants *in vivo* is necessary for determining if this poses an increased risk for a progressive course of neurological symptoms in patients harboring these mutations. Although the role of TH in the generation of neuromelanin and L-DOPA oxidation *in vivo* is not known, L-DOPA may be a better substrate for TH *in vivo*, as the enzyme appears to have a higher affinity for BH_4 for conversion of

L-DOPA than for tyrosine hydroxylation (Haavik, 1997). We were not able to show an excess of oxygen consumption compared to product formation in the enzyme reaction; or any direct evidence for production of H_2O_2 in the THD associated TH variants *in vitro*.

For PKU, a strict phenylalanine depleted diet has been the main treatment to date. However, some progress on alternative therapies has been made, based on the increasing knowledge of the molecular mechanisms of pathogenesis. Administration of BH_4 as supplementary treatment was approved in 2007 (Kuvan®, in the form of sapropterin dihydrochloride), and has been efficient for some patients, leading to an increased tolerance for dietary phenylalanine (Levy et al., 2007). BH_4 functions as a stabilizing agent (chaperone) for some variants of mutated PAH. For the BH_4-responsive PKU-patients this treatment alleviates the burden of strict diet requirement, and increases their quality of life (Blau and Erlandsen, 2004).

5.4 Regulation of AAHs

Mutations with total deleterious effect on the enzyme function are not expected in THD, as such mutations probably are not compatible with life, based on the essential role of TH in the development of the cardiovascular and nervous systems (Kobayashi et al., 1995). To date, no disease associated mutations in the AAHs in the highly conserved regulatory domain serine or threonine residues are reported. Mutations in this region will probably interfere with the intricate regulation of activity of the enzymes, and modify the phosphorylation and binding to regulatory molecules, like the 14-3-3 proteins, of the AAH (Dunkley et al., 2004; Haycock, 1990; Haycock and Haycock, 1991; Kleppe et al., 2014; Miranda et al., 2002). Interestingly, in the study of the TH R202H knock-in mouse, the enzyme displayed deficient binding to catecholamines and was not stabilized by interaction with catecholamines, as expected for TH. As previously described, the TH R202 mouse has almost normal levels of TH in the *substantia nigra*, but deficiency of TH protein in the striatum, possibly reflecting the impact of the instability of the enzyme subjected to axonal transportation. This example illustrates that mutations of subtle effect on function and regulation requires

extensive investigation to understand the pathogenetic mechanisms and provide targets for treatment.

6 Concluding remarks and future perspectives

By investigating the oxygen dependence of the AAHs, their role in hypoxic conditions, e.g. the role of TH in altitude sickness and hypoxia induced hypertension as seen in OSAS, can be better understood and provide a basis for new therapeutic strategies.

Studies of recombinant disease associated proteins, elucidating molecular mechanisms of disease, like the studies presented here, may contribute to advance individualized therapies for THD patients in the future. As current treatment of THD and Parkinson's disease with L-DOPA supplement or dopamine agonists often are unable to provide lasting and adequate symptom relief, searches for treatments that directly target the malfunctioning TH are being conducted. For PAH, the cofactor BH_4 has a stabilizing effect, mainly as a pharmacological chaperone aiding correct folding of the protein (Underhaug et al., 2012). The stabilizing effect of BH_4 for TH is less established. Searches for compounds with stabilizing effect on THD and PKU associated mutant protein have discovered a number of potential molecules; and are currently being tested on isolated enzymes, in cell culture and in animal models (Calvo et al., 2010; Hole et al., 2015; Hole et al., 2016). Different compounds had different mechanisms of protection of the activity of TH; both a classical chaperone stabilizing effect and a subgroup of compounds that preserved the activity of TH by weak binding to the catalytic site iron (Hole et al., 2015). Possibly, a synergistic effect can be achieved by combining different compounds for treatment of THD and PKU.

Combining *in vitro* studies on recombinant enzyme, protein studies in the context of intact neuronal cell systems and animal model work for analyzing disease processes, our studies can be applicable not only to AAH-associated diseases, but also to other neurogenetic disorders, in which the stability and reaction mechanisms of mutant proteins are affected. If the protein of interest is required to be active in axons and dendrites, then pharmacological chaperones that promote the stable transport of the mutant protein to these regions could provide a valuable treatment option. Thus,

therapies in which pharmacological chaperones are used to stabilize mutant proteins are currently being discussed as potential treatments for several neurogenetic disorders (e.g. lysosomal storage diseases (Parenti et al., 2015) and Friedreich's ataxia (Marmolino and Acquaviva, 2009)), in addition to the AAH related diseases discussed here.

Errata

Fossbakk, A., Kleppe, R., Knappskog, P.M., Martinez, A. & Haavik, J. (2014): "Functional studies of tyrosine hydroxylase missense variants reveal distinct patterns of molecular defects in DOPA responsive dystonia", Human Mutation 35: 7, 880-890.
Figure 2 A. The missense mutation p.Arg414Pro is placed both in exon 12 and 13, only exon 12 is correct.

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