Functional Properties of Missense Variants of Human Tryptophan Hydroxylase 2

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Communicated by Jan Kraus
Received 22 August 2008; accepted revised manuscript 21 November 2008.
Published online 24 March 2009 in Wiley InterScience (www.interscience.wiley.com). DOI 10.1002/humu.20956

ABSTRACT: Tryptophan hydroxylase 2 (TPH2) catalyzes the rate-limiting step in serotonin biosynthesis in the nervous system. Several variants of human TPH2 have been reported to be associated with a spectrum of neuropsychiatric disorders such as unipolar major depression, bipolar disorder, suicidality, and attention-deficit/hyperactivity disorder (ADHD). We used three different expression systems: rabbit reticulocyte lysate, Escherichia coli, and human embryonic kidney cells, to identify functional effects of all human TPH2 missense variants reported to date. The properties of mutants affecting the regulatory domain, that is, p.Leu36Val, p.Leu36Pro, p.Ser41Trp, and p.Arg55Cys, were indistinguishable from the wild-type (WT). Moderate loss-of-function effects were observed for mutants in the catalytic and oligomerization domains, that is, p.Pro206Ser, p.Ala328Val, p.Arg441His, and p.Asp479Glu, which were manifested via stability and solubility effects, whereas p.Arg303Trp had severely reduced solubility and was completely inactive. All variants were tested as substrates for protein kinase A and were found to have similar phosphorylation stoichiometries. A standardized assay protocol as described here for activity and solubility screening should also be useful for determining properties of other TPH2 variants that will be discovered in the future.


KEY WORDS: missense mutant; tryptophan hydroxylase; TPH2; protein stability; serotonin; phosphorylation

Introduction

Serotonin (5-HT) is an indoleamine that is widely distributed in the human central nervous system and in certain peripheral tissues. It modulates a range of physiological functions, including regulation of gastrointestinal motility, blood circulation and hemostasis, sleep, appetite, and sexual behaviours [Lucki, 1998]. The 5-HT system is evolutionarily conserved [Russo et al., 2007], and is one of the most important drug targets known in pharmacology, in particular, within psychopharmacology [Bloom and Kupfer, 1995; Chamberlaine et al., 2006]. Although a number of molecular genetic studies have been performed on different genes involved in serotonergic neurotransmission [Haddley et al., 2008; Russo et al., 2007], the precise clinical correlates of either reduced or excessive serotonergic neurotransmission are unknown. Tryptophan hydroxylase (TPH) is responsible for the 5-hydroxylation of L-tryptophan (L-Trp), which is the rate-limiting step in 5-HT biosynthesis and the first step in melatonin production [Teigen et al., 2007]. Two different TPH enzymes (TPH1 and TPH2) are expressed by two distinct genes: TPH1 (MIM 191060) appears to be mainly expressed in nonneuronal serotonergic cells, for example, skin, pineal gland, and enterochromaffin cells [Slominski et al., 2005]; whereas TPH2 (MIM 607478) is expressed in neuronal serotonergic cells, for example, serotonergic neurons of the brain and gut [Cote et al., 2003; Gutknecht et al., 2008]. The TPH enzymes belong to a superfamily of aromatic amino acid hydroxylases (AAAH). They are iron- and tetrahydrobiopterin (BH4)-dependent monoxygenases; that is, one atom of O2 is incorporated into the respective amino acid substrates, whereas the other is ultimately reduced to water [Teigen et al., 2007]. Crystal structures of the catalytic domain of all three AAAH enzymes have shown that they all possess a mononuclear nonheme iron binding motif, referred to as the 2-His-1-carboxylate facial triad, which is conserved in TPH2: His318, His323, and Glu363 [Hegg and Que, 1997; Walther and Bader, 2003; Wang et al., 2002]. Several studies indicate that the AAAH enzymes have a common catalytic mechanism, whereby iron plays a role in oxygen activation and formation of the hydroxylating intermediate [Pavon and Fitzpatrick, 2006]. Phosphorylation of the TPH enzymes modulates 14–3–3 binding: via Ser19 in TPH2 [Winge et al., 2008] and most likely via Ser58 in TPH1 [Huang et al., 2008], which corresponds to Ser104, a conserved phosphorylation site in TPH2 [Winge et al., 2008]. Serotonergic neurons and enzymatic tryptophan hydroxylation in the brain were described more than four decades ago [Dahlstrom and Fuxe, 1964; Grahame-Smith, 1964]; yet the tph2 gene was only recently identified [Walther et al., 2003]. Since then, efforts have been made in several laboratories to examine the gene for functional variants and to determine whether these variants can be correlated with aspects of human or animal behavior. As new mutations have been discovered, various functional studies have been reported [Haavik et al., 2008]. However, in most reports gene variants have been described without a functional characterization, and no laboratories have reported comparative studies of multiple TPH2 gene variants. Furthermore, conflicting effects have been reported for some mutations [Winge et al., 2007]. The purpose of this study is to systematically describe all missense mutations of human TPH2 that have been reported to date and to establish standardized assay protocols that include both structural and functional effects at the

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molecular and cellular level, for example, stability, solubility, enzyme activity, and phosphorylation stoichiometry. This is important for elucidating the mechanisms whereby missense mutations in TPH2 could affect L-Trp metabolism to 5-OH-Trp and thus the production of serotonin in the nervous system.

Materials and Methods

Source of Materials

Amylose resin for enzyme purification was purchased from New England Biolabs (Ipswich, MA). All other reagents were purchased from Sigma (St. Louis, MO). (6R)-L-erythro-5,6,7,8-tetrahydrobipterin was purchased from Shireks Laboratories (Jona, Switzerland).

Expression Vectors

Two different expression vectors were used; pETM41, for 6xHis-MBP-TPH2 expression in *Escherichia coli*, and pcDNA5/FRT, for expression of native TPH2 in a cell free expression system and in HEK293 cells, essentially as described previously [Cichon et al., 2008; McKinney et al., 2005, 2008]. Missense mutations were introduced using the Quik Change site directed mutagenesis kit from Stratagene (La Jolla, CA). Mutagenesis was done with primers from MWG Biotech (Ebersberg, Germany). Primer sequences, with their corresponding DNA, amino acid, and codon change were as follows: c.106C>G (p.Leu36Val), gctactggccagctcacaGaGTaataaatcacttgttgc; c.107C>T (p.Leu36Pro), caacttgccagctcacaACAaataaatcactgttgc; c.112C>T (p.Ala328Val), gatgcttccactacttGTG; c.118C>T (p.Asp479Glu), gaatacagtgtgtgactac; c.122G>A (p.Arg441His), gcaagttctaggtacag; c.133G>A (p.Arg55Cys), gcaacaagagggctcagatcc; c.136C>T (p.Arg303Trp), ctgtacccagtacatcTGG; c.1437T>G (p.Pro206Ser), aattatggtcagtgtggc; c.1632G>A (p.Ser41Tyr), ctaaataaacctaacTATggcaaaaatgacg; c.1760C>T (p.Arg55Cys), ggaagcagcaaaTGTgaagctgc; c.616C>T (p.Arg155Cys), gtcctccagccccttctcagtatacttcaatcc; c.907C>T (p.Ala28Val), gaagctgagggagtacctgct; c.983C>T (p.Pro328Ser), aagcagcaaaTGTgaagctgcttcccagggtg; c.988C>T (p.Ala329Val), gagatctagctatcTTGcatatctgg; c.990C>T (p.Arg329Trp), cgattcctagctatcTTGcatatctgg; c.1222G>A (p.Arg441His), gcaagttctaggtacag; c.1224G>A (p.Arg441His), gcaagttctaggtacag; c.1347T>G (p.Asp479Glu), gcataaatgcagctggctc; c.1372G>A (p.Arg441His), gcaagttctaggtacag; c.1374G>A (p.Arg441His), gcaagttctaggtacag; c.1376G>A (p.Arg441His), gcaagttctaggtacag; c.1437T>G (p.Asp479Glu), gcataaatgcagctggctc; c.1437G>A (p.Arg441His), gcaagttctaggtacag; c.1437T>G (p.Asp479Glu), gcataaatgcagctggctc; c.1437G>A (p.Arg441His), gcaagttctaggtacag; c.1437T>G (p.Asp479Glu), gcataaatgcagctggctc; c.1437G>A (p.Arg441His), gcaagttctaggtacag; c.1437T>G (p.Asp479Glu), gcataaatgcagctggctc; c.1437G>A (p.Arg441His), gcaagttctaggtacag; c.1437T>G (p.Asp479Glu), gcataaatgcagctggctc; c.1437G>A (p.Arg441His), gcaagttctaggtacag; c.1437T>G (p.Asp479Glu), gcataaatgcagctggctc; c.1437G>A (p.Arg441His), gcaagttctaggtacag; c.1437T>G (p.Asp479Glu), gcataaatgcagctggctc; c.1437G>A (p.Arg441His), gcaagttctaggtacag; c.1437T>G (p.Asp479Glu), gcataaatgcagctggctc; c.1437G>A (p.Arg441His), gcaagttctaggtacag; c.1437T>G (p.Asp479Glu), gcataaatgcagctggctc; c.1437G>A (p.Arg441His), gcaagttctaggtacag; c.1437T>G (p.Asp479Glu), gcataaatgcagctggctc; c.1437G>A (p.Arg441His), gcaagttctaggtacag; c.1437T>G (p.Asp479Glu), gcataaatgcagctggctc; c.1437G>A (p.Arg441His), gcaagttctaggtacag; c.1437T>G (p.Asp479Glu), gcataaatgcagctggctc; c.1437G>A (p.Arg441His), gcaagttctaggtacag; c.1437T>G (p.Asp479Glu), gcataaatgcagctggctc; c.1437G>A (p.Arg441His), gcaagttctaggtacag; c.1437T>G (p.Asp479Glu), gcataaatgcagctggctc; c.1437G>A (p.Arg441His), gcaagttctaggtacag; c.1437T>G (p.Asp479Glu), gcataaatgcagctggctc; c.1437G>A (p.Arg441His), gcaagttctaggtacag; c.1437T>G (p.Asp479Glu), gcataaatgcagctggctc; c.1437G>A (p.Arg441His), gcaagttctaggtacag; c.1437T>G (p.Asp479Glu), gcataaatgcagctggctc; c.1437G>A (p.Arg441His), gcaagttctaggtacag; c.1437T>G (p.Asp479Glu), gcataaatgcagctggctc; c.1437G>A (p.Arg441His), gcaagttctaggtacag; c.1437T>G (p.Asp479Glu), gcataaatgcagctggctc; c.1437G>A (p.Arg441His), gcaagttctaggtacag; c.1437T>G (p.Asp479Glu), gcataaatgcagctggctc; c.1437G>A (p.Arg441His), gcaagttctaggtacag.

Expression and Analysis of TPH2 WT and Missense Mutants Expressed in *E. coli*

The 6xHisMBP–TPH2 fusion protein was expressed in BL21-Codon Plus (DE3)-RIPL (Stratagene, La Jolla, CA) upon induction by IPTG at 20°C, essentially as described [Winge et al., 2007]. Cell pellets were homogenized in 20 mM HEPES pH 7.4, 400 mM NaCl, 10% glycerol, 10 mM benzamidine, 1 mg/mL lysosome, 1 µg/mL pepstatin A, 4.6 µg/mL leupeptin. Eluted fusion proteins were concentrated to ca. 1 mg/mL and then incubated over night at 4°C with an equivalent amount of tobacco etch virus (TEV) protease under centrifugation at 600 × g. Phosphorylation was performed as described previously [Winge et al., 2008]. Typically, a 1:10 PKA C-subunit: TPH2 ratio was used for phosphorylation. Phosphorylation stoichiometry was determined by measuring [32P] incorporation at various time points using a Tricarb 2900TR scintillation counter (Packard Bioscience, Meridan, CT) and by visualization using Phospho-Imager (Bio-Rad, Hercules, CA) after separation on 10% SDS-PAGE.

Expression and Analysis of TPH2 in Human Cells

Human embryonic kidney cells (HEK293) were transfected with the pcDNA5/FRT–TPH2 expression vector (WT and mutant forms) using lipofectamine ( Gibco, Rockville, MD) as described previously [Eiken et al., 1996; Knappskog et al., 1996]. The cells were harvested after 48 hr, pelleted, and stored at −80°C. Frozen cell pellets were thawed in 20 mM NaHepes buffer, pH 7.0 containing 1 mM orthovanadate, 0.1% Triton X-100, and Complete Protease Inhibitor Cocktail (Roche, Mannheim, Germany). Cells were homogenized by 10 passages using a 23-gauge syringe. Sucrose and NaCl were then added to a final concentration of 0.2 M and 0.15 M, respectively. The extracts were clarified by centrifugation at 12,000 × g for 10 min at 4°C. The cell pellets were homogenized in lysis buffer. The cell extracts were stored in liquid nitrogen until analyzed for enzyme levels and activity. Equal amounts (20 µg) of the soluble and pellet fractions were separated by SDS-PAGE (10%) and analyzed by Western blotting. A polyclonal mouse antihuman hydroxylase (PH8; Chemicon) at 1:2000 (v/v) was used as a primary antibody, and a goat antimouse HRP conjugate (Bio-Rad, CA) as secondary antibody. Detection was performed by chemiluminescence in a Fluor-S MultiImager (Bio-Rad) using Quantity One Software.

RNA Analysis

Total RNA was isolated from transfected cells using the RNeasy-kit (Qiagen, Hilden, Germany). Gene-specific primers, TaqMan® Minor Groove Binder (MGB) probes and Assay-on-Demand® PCR reagents were from Applied Biosystems (Foster City, CA). Quantification of mRNA was performed using the ABI 7900 instrument (Applied Biosystems). Each real-time PCR reaction contained 1 µL cDNA, 5 µL 2 TaqMan® Universal Master Mix and 0.5 µL TaqMan® primer/probe. Each sample was
run in triplicate. Cycling parameters were 95°C for 10 min, followed by 40 cycles of 95°C for 15 sec and 60°C for 1 min. Serial diluted standards were used to prepare a standard curve, which was run on the same plate and used to calculate relative gene expression abundance. GADPH was used as an endogenous control to adjust for unequal amounts of RNA. None of the samples showed signs of DNA contamination when reverse transcriptase was omitted from the cDNA reaction.

Enzyme Activity Measurements

TPH activity was assayed as described previously [Flatmark et al., 1980; McKinney et al., 2001] except for the cell free expression experiments where the BH4 concentration was 50 μM and assay time was 18 min.

Molecular Modelling/Prediction Analysis

A homology model of TPH2 (Gly48–Ile490) was prepared using the molecular modeling and drug design program: WHAT IF [Vriend, 1990], the crystal structure of double truncated TPH1 (PDB database: 1MLW) and the full-length model of TPH1 (PDB database: 1N9) [Jiang et al., 2000]. Ab initio modeling of an N-terminal domain of TPH2 (Met1–Gly48) was performed using Robetta [Kim et al., 2004]. A single representative structure was selected using the POSA Web interface [Ye and Godzik, 2005] and combined with the homology model by superimposition of Gly48 (common to both models) using the Accelrys DS Visualizer version 1.7 software (Accelrys Software Inc., San Diego, CA). The enzyme variants were analyzed for differences in atomic contacts, Van der Waals overlaps, hydrogen bonding, and salt bridges using the WHAT IF protein structure analysis software [Vriend, 1990] (Vriend group at CMBI, Nijmegen, NL).

**Results**

A literature search performed in May 2008 showed that nine different missense mutations in human TPH2 had been reported (Table 1). The minor allele frequency of the most common of these variants, that is, p.Pro206Ser and p.Ser41Tyr, were reported to be 0.02–0.03 [Cichon et al., 2008; Zhou et al., 2005a,b], but it is probably lower for the other mutations, as structural variants of TPH2 are expected to be relatively rare [Haavik et al., 2008; Sachidanandam et al., 2001]. Four missense variants: p.Ser41Tyr, p.Pro206Ser, p.Arg303Trp, and p.Arg441His have been reported in patients with clinical symptoms (Table 1). Recently, we discovered a rare missense mutation (p.Arg303Trp) in two Norwegian attention-deficit/hyperactivity disorder (ADHD) patients [McKinney et al., 2008]. The other structural variants were identified via different sequencing projects and reported without clinical data. Thus, the biochemical properties of all naturally occurring, missense variants of human TPH2 reported to date have been studied here.

**Localization of TPH2 Missense Variants in its Three-dimensional Structure**

A full-length structural model of human TPH2 was prepared by a combination of homology and ab initio modeling. Examination

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**Table 1. Features of the TPH2 Missense Mutations Included in This Study**

<table>
<thead>
<tr>
<th>Amino acid change</th>
<th>Exon</th>
<th>Nucleotide Change</th>
<th>Minor allele frequency</th>
<th>Reported with clinical symptoms</th>
<th>Enzyme domain/secondary structure/probable consequence of mutation</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Leu36Val</td>
<td>2</td>
<td>c.106C&gt;G</td>
<td>0.005</td>
<td></td>
<td>Regulatory domain/no consequence for stability, enzyme activity, or phosphorylation stoichiometry.</td>
<td>[Nielsen et al., 2008]</td>
</tr>
<tr>
<td>Leu36Pro</td>
<td>2</td>
<td>c.107T&gt;C</td>
<td>0.02</td>
<td></td>
<td>Regulatory domain/no consequence for stability, enzyme activity, or phosphorylation stoichiometry.</td>
<td>[Breidenthal et al., 2004]</td>
</tr>
<tr>
<td>Ser41Tyr</td>
<td>2</td>
<td>c.122C&gt;A</td>
<td>0.03</td>
<td>yes, bipolar disorder</td>
<td>Regulatory domain/no consequence for stability, enzyme activity, or phosphorylation stoichiometry.</td>
<td>[Zhou et al., 2005a,b]</td>
</tr>
<tr>
<td>Arg53Cys</td>
<td>2</td>
<td>c.163C&gt;T</td>
<td>n.d.</td>
<td></td>
<td>Regulatory domain/no consequence for stability, enzyme activity, or phosphorylation stoichiometry.</td>
<td>[Lin et al., 2007]</td>
</tr>
<tr>
<td>Pro206Ser</td>
<td>6</td>
<td>c.616C&gt;T</td>
<td>0.02, 0.01</td>
<td>yes, bipolar disorder</td>
<td>Catalytic domain/CoI/Pro206 is surface exposed, thus no steric hindrance observed for P206S. The Pro &gt; Ser mutation probably results in reduced conformational stability.</td>
<td>[Zhou et al., 2005a,b]</td>
</tr>
<tr>
<td>Arg303Trp</td>
<td>7</td>
<td>c.907C&gt;T</td>
<td>n.d.</td>
<td>yes, ADHD</td>
<td>Catalytic domain/CoI/Trp has larger volume, +15 Å³, which probably results in major steric overlaps with several surrounding residues, ≤1.7 Å, which probably alters conformational stability. The Arg &gt; Trp mutation also alters the net charge and probably alters substrate orientation.</td>
<td>[McKinney et al., 2008]</td>
</tr>
<tr>
<td>Ala328Val</td>
<td>8</td>
<td>c.983C&gt;T</td>
<td>n.d.</td>
<td></td>
<td>Catalytic domain/turn/Val has a larger volume, +38 Å³ but probably only results in minor steric overlaps with surrounding residues, which border the active site ≤0.35 Å. The Ala &gt; Val mutation probably decreases conformational stability and alters the active site.</td>
<td>[McKinney et al., 2001]</td>
</tr>
<tr>
<td>Arg441His</td>
<td>11</td>
<td>c.1322G&gt;A</td>
<td>0.0001</td>
<td>yes, unipolar depression</td>
<td>Oligomerization domain/loop/His has a smaller volume, ~30 Å³, but the Arg &gt; His mutation alters the net charge, which probably results in loss of a hydrogen-bond with Cα backbone oxygen of proximal residues, thus decreasing conformational stability.</td>
<td>[Zhang et al., 2005a,b]</td>
</tr>
<tr>
<td>Asp479Glu</td>
<td>11</td>
<td>c.1437T&gt;G</td>
<td>n.d.</td>
<td></td>
<td>Oligomerization domain/helix/Asp appears to form salt bridges with Lys and Arg residues in the catalytic domain of neighboring subunits. Glu conserves the net charge but has a longer side chain, +18 Å³. The Asp &gt; Glu mutation probably has decreased conformational stability due to altered subunit organization.</td>
<td>[Winge et al., 2007]</td>
</tr>
</tbody>
</table>

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See Materials and Methods for an explanation of numbering, which is based on human TPH2 cDNA (GenBank reference: NM_173353.3). The numbering starts with 1 at a ATG-start codon and is based on 11 exons with a total of 490 amino acids [Walther et al., 2003].

Reference numbers for the single nucleotide polymorphism database: www.ncbi.nlm.nih.gov/projects/SNP/, are provided when possible: c. rs4415267, d. rs17105653, e. rs2887147, f. rs7488262. New variations or details on TPH2 are updated in the Pediatric Neurotransmitter Disorder Data Base (PNDDB) database (www.bioPKU.org). ADHD, attention deficit/hyperactivity disorder; n.d., not determined.
of the model (Fig. 1 and Table 1) shows that the missense variants are distributed over all three enzyme domains. Three mutations can be localized to the catalytic domain, that is, p.Pro206Ser, p.Arg303Trp, and p.Ala328Val, which is very similar to the catalytic domain of TPH1 (83% sequence identity). None of the mutated residues are in close proximity to the active site iron: that is, the Cx atom of Pro206, Arg303, and Ala328 are 22.3, 14.4, and 15.8 Å from the active site Fe atom, respectively.

Several substitutions involve alteration in net charge (p.Arg55Cys, p.Arg303Trp, p.Arg441His) or potentially severe alterations in secondary structure (p.Leu36Pro and p.Pro206Ser) (Table 1). The mutated residues of TPH2 variants with reduced activity were analyzed for compatibility with the WT structural model to find structural explanations for their effects. The p.Arg303Trp mutant could be clearly identified as incompatible with the WT structure, that is, a charged aliphatic residue is replaced with a bulky aromatic residue, which overlaps with neighboring residues (≤1.7 Å) and the conserved Arg is also believed to play a functional role in TPH activity [McKinney et al., 2001].

The p.Ala328Val and p.Asp479Glu variants are more conservative substitutions, that is, hydrophobic for hydrophobic or charged for charged but with slightly larger volumes. Consequently, the p.Ala328Val substitution resulted in minor Van der Waals overlaps, <0.35 Å, with residues in the catalytic domain. Both Asp479 and Pro206 are found on surface-exposed side chains and might accommodate their respective substitutions without sterical hindrance. The p.Pro206Ser substitution could result in reduced stability due to loss of structural restraints imposed by the proline. The p.Arg441His and p.Asp479Glu mutations also appear to be accommodated by a tetrameric structure (not shown) but only at the cost of altered ionic interactions (Table 1).

Ab initio models of Met1-Gly48 were prepared because no suitable structural template for homology modeling exists. This N-terminal region was predicted to be relatively unstructured and includes Leu36 and Ser41 (included in this study) and Ser19, which is phosphorylated by PKA and CAMKII [McKinney et al., 2005; Winge et al., 2008]. None of the mutants were within close proximity to Ser19, that is, the Cx–Cx distance to Leu36 and Ser41 is 16.3 ± 3.7 Å and 17.7 ± 2.5 Å, respectively. Nor were any of the mutants close to Ser104, which is phosphorylated by PKA [Winge et al., 2008].

Rapid Activity Determination of TPH2 Variants in Cell Free Expression System

Using the cell free expression system, we observed that expression levels of all TPH2 variants were similar and reproducible and the enzyme variants remained in the soluble fraction after centrifugation for 30 min at 10,000 × g (Fig. 2). The specific activity was decreased for all mutations found in the catalytic and oligomerization domains of TPH2. Reduced specific activity in this system is a strong indication of reduced enzyme activity in vivo, as has been shown for inborn errors of metabolism affecting phenylalanine hydroxylase (PAH; MIM# 261600) [Knappskog et al., 1996] and tyrosine hydroxylase (TH; MIM# 191290) [Ludecke et al., 1996]. However, these experiments provide little information about the mechanisms by which the functional effects occur.

Solubility and Activity of TPH2 Variants expressed in E. coli

All variants were expressed as intact fusion proteins at high levels in E. coli (Fig. 3). They were detected by either Western blotting (results not shown) or Coomassie staining following separation by SDS-PAGE (Fig. 3). TPH activity was present in

![Figure 1](image1.png)

**Figure 1.** Molecular model of a full-length subunit of TPH2 WT, illustrating the location of missense mutants and phosphorylation sites. WT residues are shown as a ball-and-stick formation. A red ball represents the location of the active site iron atom. Arbitrarily determined domain boundaries are Met1–Asp150 for the regulatory domain, which is shown in pink; Val151–Asn448 for the catalytic domain, shown in blue, and Pro449–Ile490 for the oligomerization domain, shown in green.

![Figure 2](image2.png)

**Figure 2.** Specific activity of WT and mutant variants of human TPH2 produced using in vitro transcription and translation. TPH2 WT and mutant enzymes were expressed in rabbit reticulocyte lysates with [35S]-labeled methionine. The TPH2 specific enzyme activity of gel-filtered lysates was determined by measuring BH4-dependent 5-OH-Trp formation by HPLC with fluorometric detection in a standardized enzyme activity assay mixture. Specific activities are presented as the mean value ± SEM of at least three experiments and were adjusted for TPH2 enzyme amount by analysis of [35S] methionine-labeled TPH2 band intensity upon separation by SDS-PAGE. The activities of p.Pro206Ser, p.Arg303Trp, p.Ala328Val, p.Arg441His, and p.Asp479Glu were significantly lower than WT (p<0.05, two-tailed t-test).
both the soluble and insoluble fractions of E. coli lysates. As shown in Fig. 3, the properties of p.Ser41Tyr, p.Leu36Val, p.Leu36Pro, and p.Arg55Cys were indistinguishable from the WT on the basis of their solubility and specific activity. Mutants p.Pro206Ser, p.Arg303Trp, p.Ala328Val, p.Arg441His, and p.Asp479Glu had significantly lower residual activities (p < 0.001 t-test) in the soluble fraction, ranging from 0–80% of the WT. All loss-of-activity variants also had decreased solubility (69–79% of the WT). However, p.Asp479Glu had significantly reduced solubility but relatively intact catalytic activity.

**Rapid Purification and Phosphorylation of TPH2 Variants Expressed in E. coli**

We have observed extensive batch-to-batch variation in aggregation levels and yields of purified protein in previous experiments with TPH1 and TPH2 from E. coli [Winge et al., 2007, 2008]. Therefore, a parallel purification approach was adopted, aiming for a more robust, reproducible, and fast production of enzyme [Charbonnier et al., 2006].

All TPH2 variants were obtained as pure 6xHisMBP fusion proteins, except for p.Arg303Trp, which showed a markedly lower abundance (Fig. 4A). All purified variants were cleaved by TEV protease (Fig. 4B). The yield of cleaved p.Leu36Val, p.Leu36Pro, p.Ser41Tyr, and p.Arg55Cys was 88 ± 4% of the WT on average. Except for p.Pro206Ser, which was cleaved at levels similar to the WT, mutants with reduced solubility also had relatively more uncleaved fusion protein, that is, 100%, 24%, 36%, and 22% for p.Arg303Trp, p.Ala328Val, p.Arg441His, and p.Asp479Glu, respectively. In a study where a similar method was used, less than 50% cleavage was reported for 6xHis–TPH2 WT using the same protease [Carkaci-Salli et al., 2006], indicating that 6xHisMBP–TPH2 is not an optimal substrate for this protease.

After incubation with [32P] ATP and the catalytic subunit of cyclic AMP dependent protein kinase, WT TPH2 was rapidly phosphorylated to a stoichiometry of 1.1–1.3 phosphate/monomer (Fig. 4C). TPH2 variants with decreased solubility, that is, p.Pro206Ser, p.Arg303Trp, p.Ala328Val, p.Arg441His, and p.Asp479Glu also had a lower incorporation of [32P], that is, from 60–80% of the WT. Purified TPH2 variants were phosphorylated directly after cleavage with TEV, without further purification. Thus, small amounts of uncleaved 6xHisMBP–TPH2 fusion proteins were also phosphorylated by PKA, as shown in Figure 4C; however, free 6xHisMBP was not phosphorylated. Thus, we conclude that the previously identified phosphorylation sites Ser19 and Ser104 are accessible in all the TPH2 variants studied here, with the exception of p.Arg303Trp, which could not be detected in the soluble fraction due to its rapid aggregation. Notably, the p.Ser41Tyr mutant, which has been associated with

![Figure 3](image-url)  
**Figure 3.** Activity and solubility of TPH WT and mutant enzymes. Relative amounts of 6xHis–MBP–TPH2 fusion protein and specific activity in soluble and insoluble fractions of E. coli lysates were measured by comparing the band intensities after separation with SDS-PAGE (30 µg of total protein in each sample) and Coomassie staining as described previously [McKinney et al., 2005]. Enzyme activities were measured as described [McKinney et al., 2005]. The activity and solubility of p.Pro206Ser, p.Arg303Trp, p.Ala328Val, and p.Arg441His were significantly lower than the WT. The solubility of p.Asp479Glu was significantly lower than the WT, whereas the activity was intact (p < 0.05, two-tailed t-test).

![Figure 4](image-url)  
**Figure 4.** Rapid purification and phosphorylation of TPH2 WT and missense mutants by PKA. A: Purified 6xHis–MBP–TPH2 fusion proteins; B: TEV cleaved fusion proteins; C: PKA phosphorylated TPH2 variants. The proteins were batch-purified using amylose-bound agarose and 28 µg of each protein variant were cleaved overnight with TEV (1:1). They were thereafter phosphorylated by PKA using [32P] ATP, separated on a SDS-gel and the amount of phosphorylation was detected using a phospho-imager. The positions of molecular weight standards are indicated on the left with arrows.
bipolar affective disorder and has been proposed to have a decreased activity and an altered interaction with PKA or 14-3-3 proteins, had the same specific activity and was phosphorylated to decreased activity and an altered interaction with PKA or 14-3-3.

Expression of TPH2 Variants in Human Cells

It has been hypothesized that aggregation and loss of activity of TPH1 and TPH2 is due to limitations of the nonhomologous expression host [McKinney et al., 2004]. To provide a more native environment, the HEK293 cell expression system was selected because of its ability to express many types of neuronal proteins [Thomas and Smart, 2005]. We transiently expressed TPH2 variants in HEK293 cells and compared TPH2 mRNA and protein abundance, solubility, and specific activity in the soluble fraction.

Comparison of the relative TPH2 mRNA and protein levels showed a strong correlation, except for p.Arg303Trp, p.Arg328Val, and p.Arg441His (Fig. 5A) where lower TPH2 protein levels, relative to mRNA, were observed. These mutants may have increased rates of degradation/clearance in human cells. Similar to the cell free system, all TPH2 variants appeared to be more soluble in HEK 293 cells than in E. coli: 73–83% of the total amount of TPH2 was observed in the supernatant fraction after centrifugation at 12,000 × g for 10 min (data not shown), except for p.Arg303Trp which was mainly recovered in the insoluble fraction as shown previously [McKinney et al., 2008]. These solubility estimates are higher than previously reported for WT, p.Pro206Ser, and p.Arg441His [Carkaci-Salli et al., 2006; Cichon et al., 2008; Winge et al., 2007]. The difference is explained by the use of a more effective cell lysis procedure and avoidance of repeated freeze/thaw cycles of the cells. Freezing and thawing is a well-studied stressor of protein stability that promotes denaturation and aggregation [Bhatnagar et al., 2007; Caö et al., 2003].

The activity of the p.Arg303Trp and p.Arg441His mutants in cleared HEK293 cell lysates were 0 and 9% of the WT, respectively, that is, close to previously reported values [McKinney et al., 2008; Winge et al., 2007]. Low TPH2 protein levels in the lysates accompanied these low activities. Interestingly, when specific enzyme activities were compared with relative abundance of soluble TPH2, only p.Arg303Trp and p.Arg328Val could be clearly identified as loss-of-function variants, with 0 and 48% residual activities, respectively (Fig. 5B). The reduced TPH2 homospecific activity suggests that p.Arg303Trp and p.Ala328Val have an intrinsic loss of activity that is not explained by differences in stability and solubility alone.

Discussion

Here we present a systematic study of all missense variants of human TPH2 that have been published so far. This characterization involves a compatibility analysis of individual mutants in a full-length model TPH2 structure, as well as solubility and activity analyses in three different expression systems. Extensive information has been gathered about the structurally and functionally related enzymes: PAH and TH, which are important models for comparison of the effects of TPH2 missense mutations. In particular, the molecular pathology of PAH in phenylketonuria (PKU), an inborn error in phenylalanine metabolism, has been extensively researched such that it is an established model for protein misfolding diseases [Scrivener, 2007]. Affected individuals have a defect in the PAH enzyme and approximately two-thirds have amino acid substitutions [Pey et al., 2007; Waters et al., 2000]. The predominant molecular mechanism in PKU appears to be a loss-of-function pathogenesis due to decreased stability and/or folding efficiency of mutant PAH enzyme, which correlates well with phenotypic severity [Scrivener, 2007]. Analysis of the TPH2 variants show that the most severe mutations are found in the catalytic and oligomerization domains of the enzyme, whereas the mutations in the N-terminal regions have less impact on its catalytic function, as has been found for mutations affecting PAH and TH [Haavik et al., 2008].

The strong correlation between loss of enzyme activity and decreased solubility (Fig. 3) is in accordance with studies on TH and PAH, showing that enzyme solubility is a good predictor for an intact enzyme function. However, two of the mutants (p.Arg303Trp and p.Ala328Val) have a decreased activity that cannot be fully explained by decreased solubility, but may be related to changes in the catalytic site, and p.Asp479Glu has a relatively conserved activity despite its reduced solubility (Figs. 2 and 3). It has been reported that the N-terminal domain of TPH2 is responsible for the lower solubility and stability of TPH2 in comparison to TH and PAH [Carkaci-Salli et al., 2006; Thorolfsson et al., 2002]. However, for all three enzymes, phosphorylation of serines in the N-terminal appears to stabilize the protein [Miranda et al., 2002; Moy and Tsai, 2004; Royo et al., 2005; Winge et al., 2008]. Interestingly, mutations in this domain appear to have the least impact on enzyme stability, solubility, or
phosphorylation of TPH2. Nonsynonymous mutations may lead to mRNA instability, RNA splicing defects, or decreased translational efficiency [Chen and Miller, 2008; Weatherall, 2000]. However, we found no strong discrepancy between the amount of RNA produced in HEK293 cells for the WT and mutant TPH2, suggesting that their transcriptional efficiency or RNA stability were similar to the WT.

Recent sequencing projects have demonstrated that coding sequence variants in TPH2 are rare, as observed for most other human genes [Sachidanandam et al., 2001]. Strikingly, except for the p.Arg303Trp mutant, all the variants have some residual activity, possibly reflecting the importance of this enzyme in human health. Except for p.Arg303Trp, the homospecific activities of soluble TPH2 mutants were ≥ 60% of the WT, illustrating that TPH2 variants may have severely decreased stability and solubility but intact catalytic function.

Genotype–Phenotype Correlations

Although TPH2 recently has appeared as a promising candidate gene for several psychiatric disorders, our understanding of the role of TPH2 sequence variants in the pathogenesis of these disorders and normal human behavior is still rudimentary. Thus, the purpose of our study was to develop a robust assay system for determination of TPH2 functional properties and to illuminate the relationship between TPH2 function and psychiatric disorders. The recently published results from TPH2 knock-out mice, which show that TPH2−/− mice have no major behavioral phenotype [Gutknecht et al., 2008], illustrate the need to accumulate more clinical data, genotypes, and functional studies to define the role of this enzyme in human health.

Studies of both mendelian and multifactorial disorders have shown that different mutations within a single gene may be associated with a spectrum of clinical phenotypes. Different levels of residual activity in the mutant proteins, or the possible gain of function of some mutations, leading to additional pathogenetic mechanisms, may explain this. This could also be the case for TPH2. Thus, the p.Ser41Tyr [Lin et al., 2007; Zhou et al., 2005a,b] and p.Pro206Ser [Cichon et al., 2008] variants were associated with bipolar disorder, p.Asp414His [Zhang et al., 2005] with major depression, and p.Arg303Trp [McKinney et al., 2008] was found in two patients with ADHD, where one also reported episodes of depression and dysphoria. At first glance, the functional studies thus would indicate that mild mutations like p.Ser41Tyr and p.Pro206Ser are associated with bipolar I disorder, while mutations either affecting the active site (p.Arg303Trp) or severely reducing the enzyme stability (p.Asp414His) would be associated with symptoms of ADHD and/or depression. However, the total number of recorded patients with TPH2 mutations is too low to perform meaningful statistical analyses of such a correlation. Also, the clinical description of the patients is fragmentary, which hinders dimensional diagnoses and possible gain of function in patients with TPH2 mutations and impaired brain serotonin production. Rather, we believe that the pleiotropic clinical manifestations of TPH2 dysfunction reflect the role of serotonin deficiency as a common risk factor for several psychiatric disorders across traditional diagnostic boundaries [Russo et al., 2007].

Although the variants p.Ser41Tyr and p.Arg55Cys do not appear to interfere with the catalytic activity of TPH2, we cannot exclude that functional alterations would be detectable under different experimental conditions. On the other hand, we can be rather confident that mutations with severely disrupted stability and/or solubility using these experimental systems will also have decreased enzymatic activity in vivo.

Comparison of Different Expression Systems

The three protein expression systems that have been used here were selected to provide comprehensive information about the properties of the mutant proteins. Thus, by expressing the proteins with the stabilizing and solubilizing MBP fusion partner, we were able to show that only the p.Arg303Trp substitution had a strong effect on enzymatic activity, while the decreased activity observed in the HEK293 cells and ITT system was secondary to a decreased stability and solubility.

The particular advantages of the ITT system are that it is fast to perform and has a greater consistency in expression levels than mammalian cells. HEK293 cells have a more complete biochemical machinery for important intermolecular interactions, that is, an intact membrane system and important accessory proteins, for example, brain-specific 14-3-3 protein, which may be important for mRNA stability as well as protein stability, solubility, and activity. Producing proteins in E. coli gives the possibility to prepare large amount of pure proteins that can be used to study the direct effect of the mutations on activity, solubility, stability, and also phosphorylation. Thus, using these three systems, it is possible to study the different effects of the mutations individually and combined in a complex system. An alternative expression system would be tissue cultures of human serotonergic neurons, but even in such a system the functional effects of a given mutation would not mirror an intact nervous system and developmental and physiological effects on these cells.

Acknowledgments

Sidsel Rüise, Guri Matre, and Linda Sleire are thanked for expert technical assistance. Dr. Beat Thöny, University of Zürich, is thanked for valuable discussions.

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