Kinetic Analyses Guide the Therapeutic Decision in a Novel Form of Moderate Aromatic Acid Decarboxylase Deficiency

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Abstract Background: Aromatic amino acid decarboxylase (AADC) deficiency is a rare autosomal recessive disorder resulting in a combined dopamine and serotonin deficiency. About 50% of the cases set in the neonatal period. Here, we report an atypical clinical presentation with moderate symptoms.

Patient: At 10 months old, the patient presented paroxysmal eye movements without seizures, and feeding

difficulties which were attributed to gastroesophageal reflux. She was investigated at the age of 7 years, because of orofacial dyspraxia, hypomimie, axial hypotonia and focal segmental dystonia, bilateral ptosis, without evidence for cognitive impairment.

Results: HVA [110 nM; (reference value (rv): 202–596)] and HIAA (12 nM; rv: 87–366) decreased, OMD (520 nM; rv: 5–60) and 5-HTP (56 nM; rv: 2–16) increased in CSF. We confirmed the diagnosis of AADC deficiency because the activity in plasma was low: 4 pmol/min/ml; rv: 16–137. The kinetic analysis revealed a sixfold increase in the apparent affinity for L-dopa (4.26 mM; control = 0.71), but the $V_{\max}$ was unchanged (37.5 pmol dopamine/min/ml; control = 39.1), suggesting a modification in the substrate binding-site. Molecular analysis revealed two heterozygous mutations in the DDC gene: c1040G > A; pR347Q already described, and a novel mutation c478C > T, pR160W.

Conclusion: (1) CSF neurotransmitters metabolites suggested a moderate AADC deficiency; (2) The initial velocity saturation curve for L-dopa displayed a cooperative ligand binding behavior, in keeping with the modifications of the three-dimensional structure, induced by the amino acid substitutions (3) The treatment combination of L-dopa with pyridoxine dramatically improved the quality of life, the fatigability, and the paroxysmal eye movements.

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Abbreviations

3D	Three dimensional
3-OMD	3-Ortho-methyl dopa
5-HIAA	5-Hydroxyindolacetic acid
5-HT	5-Hydroxytryptamine
5-HTP	5-Hydroxytryptophan
AADC	Aromatic L-amino acid decarboxylase
CSF	Cerebral spinal fluid
DA	Dopamine

DDC	Aromatic L-amino acid decarboxylase gene
HVA	Homovanillic acid
L-dopa	3,4-Dihydroxyphenylalanine
MTHF	Methyltetrahydrofolate
PDB	Protein database
RV	Reference value

Introduction

Aromatic L-amino acid decarboxylase (AADC, EC4.1.128) is an essential enzyme in the metabolism of the monoamine neurotransmitters serotonin and dopamine. AADC converts both 5-hydroxytryptophan (5-HTP) into serotonin (5-hydroxytryptamine, 5-HT) and 3,4-dihydroxyphenylalanine (L-dopa) into dopamine (DA). AADC is a 54 kDa homodimeric protein, with two catalytic pockets located at the dimer interface. The AADC enzyme requires pyridoxal-5'-phosphate which is the cofactor covalently linked with the K303 residue in the active site, and subsequently bound to the substrate (Burkhard et al. 2001). The recessively inherited deficiency of AADC induces a severe neuro-metabolic disorder with developmental delay, abnormal movements, oculogyric crises, and vegetative symptoms (Brun et al. 2010; Hyland et al. 1992). The symptoms typically appear in the first months of life. The investigation of neurotransmitters in cerebral spinal fluid (CSF) normally leads to the diagnosis. Patients display a typical pattern with reduction of catabolites of the dopamine and serotonin pathways, homovanillic acid (HVA) and 5-hydroxyindol-acetic acid (5-HIAA), as well as an elevation of the precursors of dopamine and serotonin, L-dopa and 5-HTP. In addition, 3-ortho-methyldopa (3-OMD) is clearly elevated resulting from methylation of accumulating L-dopa. It was considered that this pattern was unique but a similar pattern had been described in patients with mutations in the pyridoxamine 5'-phosphate oxidase gene (Mills et al. 2005). These patients had a secondary AADC deficiency due to a defect in the synthesis of the pyridoxal phosphate cofactor. Elevated concentration of vanillic acid in urine was detected in primary and secondary AADC deficiency. Vanillic acid accumulated after transamination of 3-OMD. The diagnosis was confirmed by the enzyme activity of AADC measured in plasma. Null or very low AADC activity had been attributed to more than 30 different mutations identified in the aromatic L-amino acid decarboxylase gene (*DDC*) (Chang et al. 2004; Haavik et al. 2008; Verbeek et al. 2007). Therapeutic management was challenging. Pyridoxine, as the precursor of the AADC

cofactor, monoamine oxidase inhibitors, dopamine agonists, anticholinergics, melatonin, L-dopa, and other treatments has been used. The response to treatment was variable, but overall the outcome remains poor (Brun et al. 2010; Manegold et al. 2009).

We report a new atypical clinical presentation with moderate symptoms of AADC deficiency. We have investigated the kinetics of the enzyme in plasma. We have shown that the combined R347Q and R160W substitutions induced a low L-dopa substrate binding affinity with a normal $V_{\max}$. L-dopa has been shown to have a positive cooperative effect, in relation to the changes in the 3D-dimer structure. Consequently, L-dopa has been introduced to the initial therapy with pyridoxine and dopaminergic agonist. An excellent response to the treatment has been observed.

Patient and Methods

Biochemical Analysis

Biogenic amines pterins and methyl tetrahydrofolate were analyzed as previously described (Ormazabal et al. 2005). AADC activity was measured as previously reported (Hyland et al. 1992; Blau et al. 2002).

Molecular Analysis of the DDC Gene

Genomic DNA was extracted using the GE Healthcare Illustra DNA extraction kit. Sequencing reactions were performed using the Big Dye Terminator Cycle Sequencing kit v.3.1 (Applied Biosystems), analyzed by capillary electrophoresis on an ABI 3100 sequencer (Applied Biosystems) and sequence analyses were performed using the Seqscape software v2.5 (Applied Biosystems). The informed consent was obtained from the parents according to the Necker Hospital ethics board committee.

Computer Analysis

The kinetic parameters were determined following the selection of an appropriate curve with Kaleidagraph software. Data were fitted to the Michaelis–Menten equation (hyperbolic saturation curve when $n_H = 1$) or to the Hill equation (sigmoid saturation curve when $1 < n_H < \text{protomer number}$). The equation was:
$$V_i = \frac{V_{\max} \times [\text{L-Dopa}]^{n_H}}{K_{1/2} + [\text{L-Dopa}]^{n_H}}$$
 V_i = initial velocity, $V_{\max}$ = maximum velocity, $K_{1/2}$ = apparent affinity, $[\text{L-Dopa}]$ = L-dopa concentration, n_H = Hill coefficient.

A correlation coefficient (R^2) is displayed with the curve fit's equation and coefficients. The correlation coefficient indicates how well the calculated curve fits the original data.

The three-dimensional structure of the human AADC (residues 1–475) was modeled by comparative protein modeling methods and energy minimization, using the Swiss-Model program in the automated mode (Arnold et al. 2006; Kiefer et al. 2009; Peitsch 1995). The Sus Scrofa dopa decarboxylase (protein data base (pdb) code: 1js3) was used as a template for modeling the human AADC protein with the coordinates set at 2.25 Å. Swiss-Pdb Viewer 3.7 (<http://www.expasy.org/spdbv>) was used to analyze the structural insight into AADC mutations and to visualize the structures.

Case History

This girl was born after two healthy children to unrelated parents. Birth weight, height, and head circumference were in the normal range. She showed feeding difficulties during the first 6 months of life. Hypotonia was noticed and motor development was also delayed. She was able to sit at the age of 10 months and to walk at the age of 22 months. Speech was also delayed because of buccofacial apraxia. Since the age of 10 months, her mother described attacks of tiredness associated with paroxysmal eye movements which exhibited a diurnal variation. She was referred at 7 years old, because of the persistence of the eye movements. She followed mainstream schooling. Her composite IQ was 85 (WISC III). Bilateral ptosis and buccofacial apraxia with permanent open mouth, hypersalivation and permanent nasal obstruction were observed. A fine motor examination showed dyspraxia and dystonia triggered by movement repetition. There was no hypotonia or spontaneous abnormal movements, at this period. Clinical features were not specific for AADC deficiency. Routine plasma analysis was normal. Video EEG confirmed the absence of seizure during oculogyric crises. Glucose was 3.7 and 4.2 mmol/l in CSF and plasma respectively, with a normal ratio of 0.88, thus eliminating Glut1 deficiency. Prolactin was slightly elevated (509 mU/l, $rv < 496$). Vanillic acid (10 μ mol/mmol creatinine, $rv < 2$) and vanilpyruvic acid (48.4 μ mol/mmol creatinine, $rv = 0$) were detected in urines.

Results

CSF Neurotransmitters Analysis

Results of CSF neurotransmitters were reported in Table 1. The catabolites of DA and HT pathways, HVA and HIAA decreased whereas the precursors increased. Neopterin, Biopterin, and methyltetrahydrofolate

remained in the normal ranges. These results suggested a deficiency in AADC.

AADC Enzyme Activity and Kinetic Parameters

AADC plasma activity was measured with L-dopa concentrations saturating the normal enzyme. Results were presented in Table 1. Patient plasma activity represented 8% of the mean of the child reference range, confirming the AADC deficiency. The AADC activity was, respectively, for the father and the mother, 56% and 70% of the mean of the adult reference range. The plasma was used to measure the rate of dopamine production by increasing the concentrations of the substrate L-dopa (Fig. 1). The curves were hyperbolic for the control, the mother and the father, suggesting kinetics of Michaelis–Menten type. We have determined apparent affinity and V_{max} , which were similar to those calculated by the Lineweaver–Burk plots used by Verbeek et al. (2007). In contrast, the plot of reaction velocity versus L-dopa concentration was sigmoid for the patient, revealing a cooperative ligand binding behavior and an allosteric positive homotropic effect conforming to the Hill equation. In these conditions, the patient kinetic analysis revealed a sixfold decrease of the L-dopa apparent affinity, but the V_{max} value was not decreased, suggesting an alteration of the substrate binding-site. The sigmoid curve allowed us to determine a Hill coefficient n_H of 1.78. The Hill coefficient quantifies the cooperativity, with a range between 1 and the number of protomers ($1 < n_H < \text{number of protomer}$). For control and parents, there was no cooperativity because $n_H = 1$. For the patient, the value of 1.78 indicated a positive cooperative fixation of the substrate L-dopa into the active site, in keeping with $n_H < 2$ because the number of protomers or subunits for AADC is 2.

DDC Gene Analysis

Molecular analysis of the DDC gene identified two heterozygous mutations: c1040G > A; pR347Q already described, and a novel mutation c478C > T, pR160W. The father was heterozygous for the pR347Q mutation and the mother was heterozygous for the pR160W mutation.

In Silico Analysis of the AADC Dimer 3D-Structure

The 87% homology between human and pig AADC allowed us to use the pig protein database 1JS3 as a matrix to model the human AADC, with a good precision. The mis-sense mutations pR347Q and pR160W and the interacted amino acids were conserved in the related species. In Fig. 2, the two peptide chains of the homodimer were shown as strands of ribbons for the α -helix, but only one active site has been indicated to clarify the figure. Both

Table 1 (A) CSF neurotransmitter analysis before and after 1 month treatment with L-Dopa + bromocriptine + pyridoxal phosphate treatment (nmol/L). (B) Plasma AADC enzyme activity. Before treatment CSF neurotransmitters analysis has oriented and the low plasma

AADC activity has confirmed the diagnosis of AADC. Plasma AADC activity was measured with saturating L-dopa concentrations for normal enzyme

A: Patient CSF analysis (nmol/L)	Before treatment	After treatment	Reference values
5-HTP	56	24	2-16
HIAA	12	18	87-366
3-OMD	520	794	5-60
HVA	110	123	202-596
MHPG	36	35	13-68
MTHF	100	57	>44
B: AADC activity (pmol/min/ml)			Reference values
Patient	4		16-130 < 15 y
Mother	15		14-41 > 15 y
Father	19		14-41 > 15 y
Control	24		14-41 > 15 y

Fig. 1 Plot of reaction velocity versus L-dopa concentrations. Saturation curves were fitted to the Michaelis–Menten equation or to the Hill equation, by using the equation below. Curves were hyperbolic for control, mother and father conforming to $n_H = 1$.

Curve was sigmoid for the patient, conforming to $n_H = 1.78$ ($n_H =$ Hill coefficient). The kinetic parameters were derived by nonlinear least square analysis by using the program kaleidagraph

mutations led to the loss of an arginine residue, both these arginines were located at the interface of the monomers. We analyzed how the mutations could induce modifications in salt-bridge, hydrogen, and Van der Waals contacts, with the environmental amino acids.

We showed that the loss of an arginine (mutation pR347Q) might have relaxed the catalytic pocket. The NH1 primary amine group in arginine 347 was 3.12 Å away from the oxygen of the phenylalanine 103 located into the opposite monomer. The phenylalanine 103 was in

Fig. 2 Structural impact of the AADC mutations. AADC was a homodimer with two catalytic pockets lining at the dimer interface. The human AADC was represented in *yellow* and superimposed with the pig dimeric enzyme in *gray* (pdb code 1JS3). Only one active site was shown with the substrate (L-dopa or 5-HTP in *fuchsia*), bound to the cofactor pyridoxal-5'-phosphate (in *pea green*), itself covalently linked with K303 (in *purple*). **(a)**: control; **(b)**: patient with the two substitutions located at the interface of the monomers. **(a)**: the substitution of R347 (pR347Q) is shown in *red* and led to a relaxation of the catalytic pocket by the loss of the interaction between F103 in

black and R347. The substitution of the R160 (pR160W shown in *blue*) suppressed one or another hydrogen contact and salt-bridge interaction, respectively, with G202 (in *green*) or I201 (in *pink*) and E181 (in *amber*) or E196 (in *khaki*). The interacting amino acids were located into the opposite monomer of R160. **(b)**: the introduction of W160 reinforced the hydrophobic patch shown in *black* (F237, F238, and W267) by stronger inter chains Van der Waals contacts, rendering this region less flexible. Thus, both mutations have locally altered the protein dimerization and have modified the kinetic character for its substrate L-dopa

Van der Waals contact with the catechol ring of the substrate, as reported by Burkhard et al. (2001). Thus, arginine 347 participated to the substrate fixation via its interaction with Phe103. In the mutated AADC, the catalytic pocket might be relaxed by the loss of the Phe103/R347 interaction. Furthermore, the introduction of

a tryptophane in position 160, instead of an arginine (pR160W) modified the 3D-structure. First, the NH1 primary amine group in arginine 160 was 2.85 Å away from the oxygen in glycine 202. The NH2 primary amine group in arginine 160 was 3.04 Å away from the oxygen in isoleucine 201 (Fig. 2a). The loss of arginine 160

suppressed one or another hydrogen contact with glycine or isoleucine located into the opposite monomer, resulting in a local destabilization of the dimer structure.

Second, the distances from arginine160 to glutamate181 and to glutamate 196 were 14 Å and 15 Å, respectively. They exceeded over the limit authorized to establish salt–bridge interaction in the fixed crystallized structure. However, one could postulate that the movements of the monomers reduced the distance, allowing temporary interactions for local stabilization of the dimer structure. The loss of arginine160 suppressed the salt–bridge interaction with glutamate 181 or glutamate 196, increasing the local destabilization of the dimer structure. Third, the mutation pR160W introduced the highly hydrophobic aromatic tryptophane which can establish new Van der Waals contacts. We have identified three other hydrophobic aromatic amino acids in the region, phenylalanine 237 and 238, and tryptophane 267, each being located less than 7 Å from the mutation pR160W. These three aromatic amino acids constituted a patch of hydrophobic aromatic residues between the two subunits. The introduction of the tryptophane 160 reinforced the hydrophobic patch and may have closed the access to the active site by strong inter-chains Van der Waals contacts.

Drug Therapy

As soon as the AADC deficiency was confirmed, the patient was treated two times a day with pyridoxine 125 mg and bromocriptine (D1 and D2 dopamine agonist) to a total of 0.45 mg/kg/day (7.5 mg/day). After 48 h, improvement was observed with a reduction of oculogyric crises as shown in Table 2. Dyspraxia, fatigability, and quality of life were also improved after the first month of therapy. Based on in vitro kinetic analysis, L-dopa (4 mg/kg/day) was progressively added to a dose of 10 mg/kg/day, and the patient showed an excellent response. After 6 months of combined therapy, Bromocriptine with L-dopa and pyridoxine, Bromocriptine was stopped and L-dopa was only combined with pyridoxine. Clinical improvements still increased: oculogyric crises and dystonia disappeared, and fatigability decreased, as described in Table 2. After 3 months of treatment (2 months after the initial therapy and 1 month after L-dopa supplementation), CSF neurotransmitters were analyzed again (Table 1). HIAA and HVA levels slightly increased, although the clinical response was excellent.

Discussion

AADC converts L-dopa to DA and 5-HTP to 5-HT and deficiency of this enzyme leads to profound alterations in central and peripheral nervous system homeostasis. Here, we report an atypical patient with moderate clinical features

diagnosed at 7 years old, by the analysis of neurotransmitters in CSF and the AADC enzyme activity in plasma. The patient was heterozygous for a mis-sense mutation pR347Q already described by Pons et al. (2004) and a novel mis-sense mutation pR160W.

CSF HVA level in the index patient was only 50% of the lower limit of the reference range and AADC activity using L-dopa as substrate was higher than activities reported in previous AADC deficiencies (Verbeek et al. 2007). These results were in accordance with the moderate phenotype observed in the patient. The pR347Q mutation led to a severe phenotype with null plasma AADC activity, when it was associated with a mutation pR358H in one patient or with a single base deletion in exon2 (delC209-211) in another patient as previously reported by Pons et al. (2004). In contrast, the mutations pR347Q in combination with the novel pR160W mutations led to a moderate phenotype. CSF HIAA level represented 12% of the lower limit of the reference range. It would have been of great interest to determine whether the discrepancy was also reflected in the enzyme activity toward 5-HTP as a substrate. Unfortunately, the whole plasma sample available before therapy was used for three independent kinetic analyses toward L-dopa as a substrate. Both substrates competed for the same active site, and AADC activity was 8–12 lower with 5-HTP than with L-dopa (Verbeek et al. 2007). Consequently, we postulate that the enzyme activity should be undetectable with 5-HTP as a substrate, keeping in mind the low level of HIAA.

We demonstrated a Michaelis–Menten-type kinetic with a hyperbolic curve for enzyme activity using L-dopa as a substrate in plasma of control and parents. The apparent affinity and $V_{\max}$ were in the same order of magnitude as previously described (Verbeek et al. 2007). Thus, one heterozygous mutation, either pR347Q or pR160W did not induce any modification in the kinetic parameters. In contrast, the plot of reaction velocity versus L-dopa was sigmoid for the patient, conforming to the Hill equation with $n_h = 1.78$. Thus, we revealed a cooperative ligand binding behavior and its allosteric positive homotropic effect. L-dopa facilitated its own fixation, by inducing a trans-conformation of the enzyme. The L-dopa apparent affinity decreased by sixfold and the $V_{\max}$ was similar to the control at saturating concentrations. The homodimeric structure was compatible with the value of 1.78 for n_h for allosteric enzyme. There was no change after dialysis, eliminating the hypothesis of an allosteric inhibitor in the plasma patient (data not shown). In the plasma, the cooperative effect resulted from a mix of three types of dimer: 50% with the combined mutations, one on each monomer, 25% with the pR347Q mutation on each monomer and 25% with the pR160W mutation on each monomer. The influence of each substrate and of the cofactor pyridoxal-5'-phosphate could be determined in

Table 2 Follow-up of the AADC therapy. Timing of tiredness attack and ocular revulsion before, after bromocriptine (0,45 mg/kg/day) + pyridoxine (125 mg two times a day) and after L-dopa (10 mg/kg/day) + pyridoxine (125 mg two times a day)

Before treatment													
	9 h	10 h	11 h	12 h	13 h	14 h	15 h	16 h	17 h	18 h	19 h	20 h	21 h
Day 1				Y	Y	Y	Y	Y				Y	
Day 2					Y							Y	
Day 3									Y			Y	
Day 4				Y					Y				
Day 5				Y									
Day 6									Y		Y	Y	
Day 7												Y	
Day 8		Y	Y	Y					Y	Y	Y		
Day 9													
Day 10				Y	Y			Y	Y			Y	
After Bromocriptine 0.45 mg/kg/day + Pyridoxine 125 mg two times a day													
	9 h	10 h	11 h	12 h	13 h	14 h	15 h	16 h	17 h	18 h	19 h	20 h	21 h
Day 1				Y	Y				Y				
Day 2													
Day 3									Y	Y	Y		
Day 4									Y		Y		
Day 5									Y		Y		
Day 6											Y		
Day 7													
Day 8									Y				
Day 9													
Day 10				Y							Y		
After 6 month L-dopa (10 mg/kg/day) + Pyridoxine 125 mg two times a day													
	9 h	10 h	11 h	12 h	13 h	14 h	15 h	16 h	17 h	18 h	19 h	20 h	21 h
Day 1													
Day 2													
Day 3													
Day 4													
Day 5													
Day 6													
Day 7													
Day 8													
Day 9													
Day 10													

Y = ocular revulsion and red squares = tiredness periods

a future study by producing recombinant wild-type and mutated AADC proteins. The combination of the three subunit types should allow to simulate the kinetic observed in the patient's plasma.

The active site is located at the monomer–monomer interface. The combination of the two mutations lowered the substrate binding affinity: the pR347Q mutation relaxed the catalytic pocket by the loss of the F103/R347 interaction, and the pR160W mutation introduced a highly hydrophobic aromatic amino acid reinforcing a strong hydrophobic patch

already present at the interface of the two monomers. Both mutations destabilized the local 3D-structure and locked the accessibility of the substrate into the binding pocket by inter chains Van der Waals contacts. Increasing the substrate concentrations resulted in a homotropic cooperative effect conforming to allosteric enzymes.

Treatment was initiated with the D1 and D2 dopamine agonist bromocriptine together with pyridoxine. Pyridoxine is the most common drug used in AADC deficiency (71% of the patients) (Brun et al. 2010), because it is the

precursor of the cofactor pyridoxal 5'-phosphate, which may play a role of chaperone molecule and stabilizes the mutated enzyme. Since we had identified a homotropic cooperative effect in vitro, we have introduced L-dopa in the therapy. Clinical improvements still increased even after stopping bromocriptine. The therapeutic use of the substrate L-dopa is less frequent (in only 13% of the patients), and the majority of cases showed none or poor response, probably because the mutations removed most AADC enzyme activity. Three siblings with the homozygous pG102S responded on L-dopa + pyridoxine (Chang et al. 2004). The mutation lied very close to the substrate binding pocket between Isoleucine 101 and phenylalanine 103. In our index case, the mutation pR347Q suppressed the R347/F103 interaction. Moreover, the second pR160W mutation introduced a strong hydrophobic patch at the interface of the two monomers reducing the accessibility of the substrate into the binding pocket. Although the homozygous mutation pG102S clearly reduced the L-dopa substrate binding affinity (apparent affinity was about 20-fold higher than the wild-type apparent affinity in plasma), there was, however, no cooperative effect and the $V_{\max}$ still remained substantially below the control level. In contrast, the $V_{\max}$ was restored with saturating amounts of L-dopa for our patient, providing the best clinical response to a mixture of L-dopa + pyridoxine. Taking into account the role of the two heterozygous mutations on the 3D-structure, it was the mix of both L-dopa and pyridoxine who probably led to an excellent clinical response and to a drop of CSF 5-HTP after therapy, since they were tested all together. However, we still have to compare the long-term follow-up therapy by L-dopa and pyridoxine for these patients.

In conclusion, we reported a moderate AADC deficiency. The clinical features were nonspecific, indicating that neurotransmitter disorders should be considered in any patients presenting abnormal ocular movements associated with autonomic dysfunction. CSF neurotransmitter analysis has led to the diagnosis. Then, AADC enzyme activity confirmed the diagnosis for a mild clinical presentation with a residual plasma activity. The two amino acid substitutions located at the interface of the monomers induce the L-dopa allosteric positive homotropic effects in keeping with the Hill equation and the modifications of the 3D-structure. The decision to supplement the treatment with L-dopa + pyridoxine led to an excellent clinical response.

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