Positive charge intrinsic to Arg^{37}-Arg^{38} is critical for dopamine inhibition of the catalytic activity of human tyrosine hydroxylase type 1

Akira Nakashima[a], Nobuhiro Hayashi[b], Keiji Mori[a], Yoko S. Kaneko[a], Toshiharu Nagatsu[c], Akira Ota[a,*]

[a] *Department of Physiology, School of Medicine, Institute for Comprehensive Medical Science, Fujita Health University, Toyoake, Aichi 470-1192, Japan*
[b] *Division of Biomedical Polymer Science, Institute for Comprehensive Medical Science, Fujita Health University, Toyoake, Aichi 470-1192, Japan*
[c] *Division of Molecular Genetics, Institute for Comprehensive Medical Science, Fujita Health University, Toyoake, Aichi 470-1192, Japan*

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Abstract Tyrosine hydroxylase (TH), which converts L-tyrosine to L-3,4-dihydroxyphenylalanine, is a rate-limiting enzyme in the biosynthesis of catecholamines; its activity is regulated by the feedback inhibition of the catecholamine products including dopamine. To rationalize the significant role of the N-terminal sequence Arg^{37}-Arg^{38} of human TH type 1 (hTH1) in determining the efficiency of feedback inhibition, we produced mutants of which the positively charged Arg^{37}-Arg^{38} site was replaced by electrically neutral Gly and/or negatively charged Glu and analyzed the degree of inhibition of these mutant enzymes by dopamine. The replacement of Arg by Gly reduced the inhibitory effect of dopamine on the catalytic activity measured in the basic pH range and the replacement of Arg by Glu was enough to abolish the inhibitory effect, although these mutations brought no significant changes to the circular dichroism spectrum. The prediction of the secondary structure of N-terminal residues 1–60 by computer software specified the location of the Arg^{37}-Arg^{38} sequence in the turn intervening between the two α-helices (residues 16–29 and residues 41–59). These results suggest that the positive charge of the amino acid residues at positions 37 and 38 is one of the main factors that maintains the characteristic of the turn and is responsible for the enzyme inhibition by dopamine.

Key words: Human tyrosine hydroxylase type 1; Dopamine inhibition; Site-directed mutagenesis; Regulatory domain; Secondary structure; N-terminus

1. Introduction

Tyrosine hydroxylase (TH; tyrosine 3-monooxygenase; L-tyrosine, tetrahydropterin: oxygen oxidoreductase (3-hydroxylating); EC 1.14.16.2) is the rate-limiting enzyme in the biosynthesis of catecholamines [1] and catalyzes the conversion of L-tyrosine to L-3,4-dihydroxyphenylalanine (L-dopa) [2]. TH consists of a catalytic domain and a regulatory domain [3]. The catalytic domain is located at the C-terminal two-thirds of the molecule and binds the substrates (L-tyrosine and molecular oxygen) and the cofactor (6*R*-tetrahydrobiopterin; $6RBPH_4$). In contrast, the important roles controlling the enzyme activity have been assigned to the N-terminal end as the regulatory domain.

The catalytic activity of TH is inhibited by the end-product catecholamines [2], and the phosphorylation of Ser residues in the N-terminus relieves the catecholamine-mediated inhibition [4]. Many observations have been reported that indicate the importance of residues 30–40 in the N-terminus in this inhibition, and Arg^{37}-Arg^{38} was found to be a key sequence for dopamine to exert its inhibitory effect on the catalytic activity of human TH type 1 (hTH1) [5,6]. The phosphorylation of Ser^{31} and Ser^{40} among three different phosphorylation sites in the N-terminus enhanced the catalytic activity of TH [7,8]. hTH3, which possesses an additional 27 amino acids between the Met^{30} and Ser^{31} of hTH1, was about five times more active than hTH1 [9]. However, all these observations concerning the N-terminus of hTH1 remain to be explained. In this study, from among the issues maintained above, we addressed the phenomenon of dopamine inhibition of hTH1 catalytic activity and sought to rationalize the critical role of the sequence Arg^{37}-Arg^{38} underlying the dopamine-induced inhibition.

In the study described herein, at first we analyzed the role of the sequence Arg^{37}-Arg^{38} in dopamine-mediated inhibition by using hTH1 mutants in which Arg^{37} and/or Arg^{38} was replaced by Gly and/or Glu to modulate their electrical charges, and, next, we predicted the secondary structure of N-terminal residues 1–60 with the assistance of computer software. Finally, we discussed the importance of the sequence Arg^{37}-Arg^{38} for the residues 30–40 in the N-terminus of hTH1.

2. Materials and methods

2.1. Materials

Escherichia coli strain BL21(DE3) (F, omp T, r^-, m^-) was purchased from Novagen (Madison, WI, USA). pKF18k vector was from Takara (Kyoto, Japan). Restriction enzymes were obtained from New England BioLabs (Beverly, MA, USA). $6RBPH_4$ was synthesized as previously described [10]. All other reagents used in this study were purchased from Sigma (St. Louis, MO, USA), Boehringer-Mannheim (Mannheim, Germany), Merck (Darmstadt, Germany), Wako (Osaka, Japan) and Nacalai (Kyoto, Japan), and were of analytical grade.

*Corresponding author. Fax: (81)-562-93 8831.
E-mail: aota@fujita-hu.ac.jp

Abbreviations: TH, tyrosine hydroxylase; hTH1, human TH type 1; RR-RG, RR-GR, RR-GG and RR-EE, mutants of hTH1 with Arg^{37}-Arg^{38} replaced by Arg^{37}-Gly^{38}, Gly^{37}-Arg^{38}, Gly^{37}-Gly^{38} and Glu^{37}-Glu^{38}, respectively; T-buffer, 20 mM Tris–HCl buffer (pH 7.3) containing 8% sucrose and 1 mM dithiothreitol

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2.2. Site-directed mutagenesis

The full-length hTH1 cDNA cloned into pET3c vector [11] was digested with *Nde*I and *Bam*HI, and the resulting hTH1 cDNA fragment was inserted into the pKF18k vector. Site-directed mutagenesis was performed with the pKF18k vector containing hTH1 cDNA as a template by the oligonucleotide-directed dual amber method [12] using a mutagenesis kit (Takara). The mutants with replacement of Arg^{37}-Arg^{38} by Gly or Glu are referred to as RR-RG for Arg^{37}-Gly^{38}, RR-GR for Gly^{37}-Arg^{38}, RR-GG for Gly^{37}-Gly^{38}, and RR-EE for Glu^{37}-Glu^{38}. The mutagenic primers used were as follows: 5′-CTC-GAT-GAG-GCT-CTG-CCT-GCC-CCC-AAT-GAA-CCG-CGG for RR-GR, 5′-CTC-GAT-GAG-GCT-CTG-CCC-GCG-CCC-AAT-GAA-CCG-CGG for RR-RG, 5′-CTC-GAT-GAG-GCT-CTG-CCC-GCC-CCC-AAT-GAA-CCG-CGG for RR-GG and 5′-CTC-GAT-GAG-GCT-CTG-CTC-CTC-CCC-AAT-GAA-CCG-CGG for RR-EE. DNA sequences of positive mutant clones were confirmed by using a model 373A DNA Sequencing System (Applied Biosystems, Foster City, CA, USA). The mutated hTH1 cDNA in the pKF18k vector was digested with *Nde*I and *Bam*HI, and the resulting hTH1 cDNA was inserted by ligation into the pET3c vector. The plasmid DNA was used to transform *E. coli* BL21(DE3).

2.3. Expression and purification of enzymes

According to the method described by Nasrin et al., the expression of the hTH1 molecule in *E. coli* strain BL21(DE3) was initiated in Luria-Bertani medium containing ampicillin and ferrous ammonium sulfate by the addition of isopropyl-β-D-thiogalactopyranoside [13]. The purification was performed at 4°C according to the method outlined by Oka [14]. Solid ammonium sulfate was added to the supernatant of the cell lysates of *E. coli* BL21(DE3) to 20% saturation, and the mixture was stirred for 1 h. The precipitate was removed by centrifugation at $15\,000\times g$ for 15 min. Then, solid ammonium sulfate was added to the supernatant to 40% saturation, and the mixture was stirred for another 1 h. The precipitate was collected by centrifugation at $15\,000\times g$ for 15 min and dissolved in 20 mM Tris–HCl buffer (pH 7.3) containing 8% sucrose and 1 mM dithiothreitol (T-buffer). Next, the solution was passed through a Superose 6HR 10/30 column (Amersham Pharmacia Bioteck, Uppsala, Sweden) equilibrated with T-buffer. The active fraction was applied to a heparin-Sepharose column (Amersham Pharmacia Bioteck) equilibrated with T-buffer, and then hTH1 protein was eluted with a gradient of KCl in T-buffer (0–0.4 M).

2.4. Assay of catalytic activity of enzymes

The catalytic activity of the mutant enzymes was assayed according to the method by Nagatsu et al. [15] with minor modifications. The enzymes were preincubated at 30°C for 20 min in 25 μl of T-buffer containing with various doses of dopamine. The concentration of each enzyme was 16 μg/ml in the preincubation solution. The assay was started by the addition of 175 μl of the mixture of 57 mM HEPES, 114 mM β-mercaptoethanol, 0.23 mg/ml catalase, 0.057 mM L-tyrosine and 0.23 mM $6RBPH_4$ to the preincubation solution, and then the sample was incubated at 37°C for 10 min in the air. The resulting L-dopa was isolated by use of a high performance liquid chromatography apparatus (Shimadzu, Kyoto, Japan) equipped with a Nucleosil $7C_{18}$ column (4.6 in diameter×250 mm) (GL Sciences, Tokyo, Japan) equilibrated with 0.1 M sodium phosphate buffer (pH 3.5) containing 1% methanol and 4 μM EDTA, and measured with an electrochemical detector ECD-100 (EiCOM, Kyoto, Japan). The assay was linear for at least 15 min at the enzyme concentration used.

2.5. Prediction of the secondary structure

The secondary structure of wild-type hTH1 was predicted from the amino acid sequence according to both the method of Chou–Fasman (CF-Pred) [16] and that of Robson–Garnier (GOR-Pred) [17] on a SPARC workstation with a Sun operating system (Sun Microsystems, Palo Alto, CA, USA) using the Sequence Analysis Software Package (Genetics Computer Group, Madison, WI, USA) [18]. The secondary structural predictions were also made according to a modified method of Robson–Garnier (modified GOR) [19], to a method using a profile-fed neural network system from Heidelberg (PHD) [20–24], and to that using the 3D-1D compatibility algorithm (3D-1D) [25,26].

2.6. Circular dichroism (CD) spectrometry

CD spectra of the wild-type hTH1 and four mutants were recorded at 25°C by using a J-720 CD spectropolarimeter (JASCO, Tokyo, Japan) equipped with a 0.1 cm cell.

2.7. Other methods

Protein concentration was measured with the Bio-Rad Protein Assay kit (Hercules, CA, USA) with bovine serum albumin as a standard. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) was performed with 12.5% gel [27] and then, the proteins were stained with Coomassie brilliant blue R-250.

3. Results

3.1. Prediction of the secondary structure

Using five different methods, we predicted the secondary structure of the N-terminus of hTH1 (residues 1–60). There

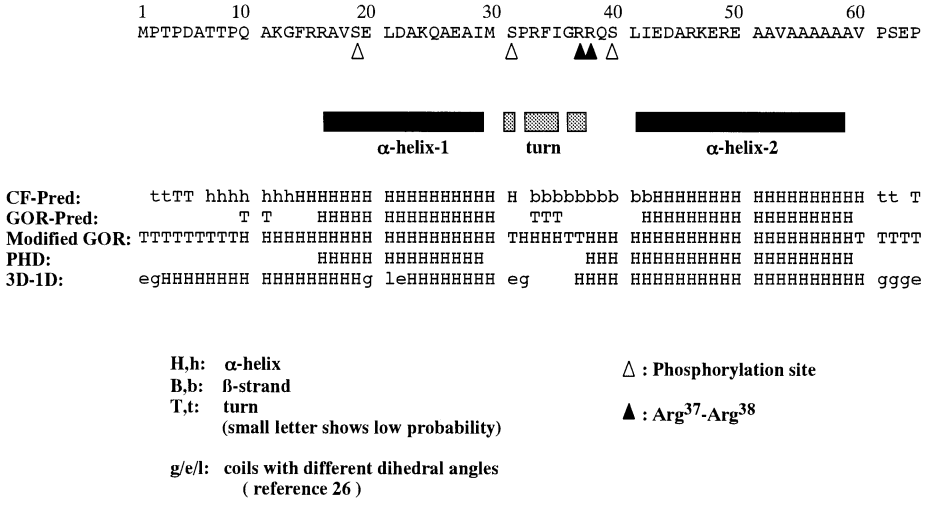

Fig. 1. Prediction of the secondary structure of the N-terminal 1–60 amino acids of hTH1. Prediction methods used were as follows: CF-Pred: the method of Chou–Fasman, GOR-Pred: the method of Robson–Garnier, Modified GOR: a modified method of Robson–Garnier, PHD: a method using a profile-fed neural network system from Heidelberg, 3D-1D: a method using the 3D-1D compatibility algorithm. Computer software used in this study is listed in the text. Open triangles indicate sites for phosphorylation by kinases; Ser^{19}: type II calcium/calmodulin-dependent protein kinase (Ca/CAM-PKII), Ser^{31}: mitogen-activated protein kinases, Ser^{40}: cyclic AMP-dependent protein kinase, protein kinase C and even Ca/CaM-PKII. Closed triangles indicate the specific portion critical for the dopamine-induced inhibition.

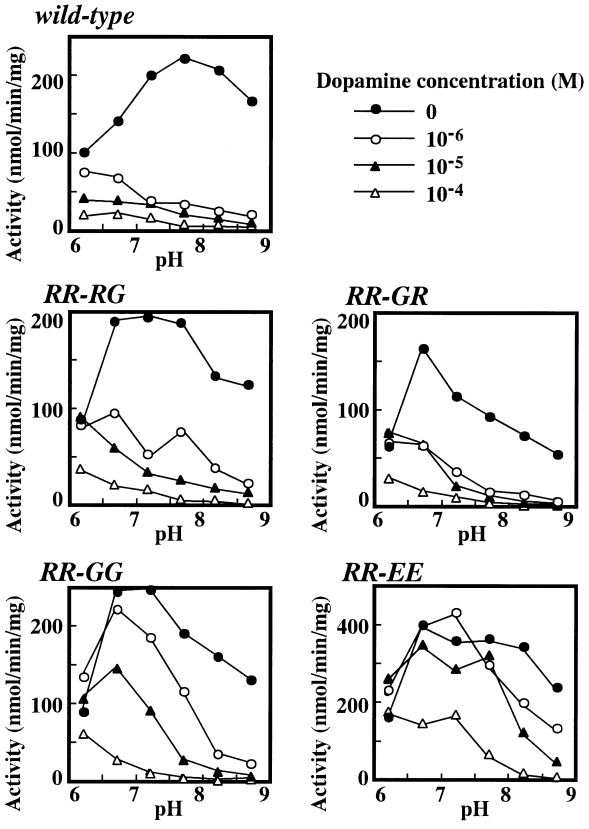

Fig. 2. Effect of preincubation with dopamine on the catalytic activity of the enzymes. The representative plots obtained from wild-type hTH1 and the four mutants are displayed in the panels. The assay conditions are described in the text. The final concentrations of L-tyrosine and 6RBPH$_4$ were 0.05 mM and 0.2 mM, respectively. Each point in the figures is expressed as the mean value of duplicate measurements.

was no significant difference among the five predictions of the secondary structure, as shown in Fig. 1. Residues 1–60 were supposed to be composed of two α-helices connected by a turn. The first α-helix corresponded to approximately amino acid residues 16–29, and the second α-helix to around amino acid residues 41–59.

3.2. Purification and TH activity

The wild-type enzyme and four mutants were purified by the following three steps: ammonium sulfate precipitation, gel-permeation with Superose 6HR 10/30 column, and heparin-Sepharose column chromatography. The elution of wild-type and the mutants from heparin-Sepharose with a gradient of KCl depended on the charge of amino acids at positions 37 and 38; wild-type, RR-RG, RR-GR, RR-GG and RR-EE eluted at the concentration of 0.36, 0.35, 0.35, 0.31 and 0.28 M KCl, respectively. The eluates of wild-type and of the mutants gave a major band with a M_r of about 59 kDa on SDS-PAGE. Their TH specific activities for the conversion of L-tyrosine to L-dopa were about 600 nmol/min/mg of protein in the assay buffer (pH 7.7) containing 0.2 mM L-tyrosine and 1 mM 6RBPH$_4$.

3.3. Inhibition by dopamine

The pH activity profiles of wild-type and four mutants were examined by use of the assay buffers with various pHs of 6.2, 6.7, 7.2, 7.7, 8.2 and 8.7 (Fig. 2: horizontal abscissa are plotted according to the actual pH values of the assay mixtures). The maximal activity of the wild-type enzyme was observed over a broad basic range. Preincubation of the wild-type with 1, 10 or 100 μM dopamine lowered the catalytic activity measured in the basic pH range. However, the strong inhibitory effect of dopamine on the catalytic activity was not observed in the acidic pH range for either wild-type or any mutants. Replacement of Arg at positions 37 and 38 by Gly reduced the inhibitory effect of dopamine on the catalytic activity measured in the basic pH range. For the mutants RR-RG and RR-GR, 100 μM dopamine inhibited the catalytic activities more strongly than 1 or 10 μM dopamine. Dopamine at 1 or 10 μM failed in inhibiting sufficiently the catalytic activity of RR-GG. For the mutant RR-EE, in which Arg was replaced by Glu, neither 1 nor 10 μM dopamine inhibited the catalytic activities. Collectively, the inhibitory effect of dopamine depended on the charge of amino acids at positions 37 and 38.

3.4. CD spectrum

Although slight differences in the overall signal strength of the CD spectra were observed among wild-type and the four mutants, the overall shapes of the CD spectra were not different from each other (data not shown).

4. Discussion

We recently reported that Arg^{37}-Arg^{38} within the N-terminus was a key sequence for the inhibitory effect of dopamine [5,6]. To clarify why the Arg^{37}-Arg^{38} sequence is so definitive in the dopamine inhibition, we thought that information on the tertiary structure of the N-terminus should be applied. However, there has been no information concerning the tertiary structure of N-terminus, because the crystallization of the TH molecule had been performed with rat TH having up to 155 amino acid residues deleted from the N-terminus [28,29]. Therefore, to rationalize the critical role of Arg^{37}-Arg^{38}, we predicted the secondary structure of residues 1–60 of the N-terminus of hTH1 with the assistance of computer software. All the predictions performed in this research converged to give the following secondary structure: the first α-helix (α-helix 1, around residues 16–29) and the second α-helix (α-helix 2, around residues 42–59) are connected by a turn (around residues 30–41) (Fig. 1). The amino acid residues Arg^{37}-Arg^{38} highlighted in this study are located in the turn connecting the two helices. It is noteworthy that the catalytic activity of hTH1 having no less than 29 amino acid residues of its N-terminus deleted was reduced to 20% of that of the wild-type enzyme [6]. The presence of α-helix 1 thus appears to be fundamental to maintain the catalytic activity of the enzyme.

It has been suggested that the characters of the amino acid residues residing in the N-terminus of TH affect the catalytic activity. Birman et al. reported that the group of negative charges formed in the N-terminus of *Drosophila* TH type II had the same effect on catalytic activity as that of certain polyanions such as heparin [30]. According to this line of evidence, we focused on the positive charges of Arg^{37}-Arg^{38} in the N-terminus of hTH1. We produced the hTH1 mutants in which Arg^{37}-Arg^{38} was replaced by Gly and/or Glu to modulate their electrical charges. Each mutant eluted at a

different concentration of KCl from heparin-Sepharose almost in parallel with the intensity of the positive charges intrinsic to the amino acids at positions 37 and 38. However, the overall shapes of the CD spectra were not different among wild-type and the four mutants in spite of the presence of the slight differences in the overall signal strength of the CD spectra from each other. Furthermore, all the mutant enzymes examined in this study possessed catalytic activity to almost the same degree as the wild-type enzyme when assayed in the dopamine-free condition. These results suggest that the mutations did not cause any significant collapse of the tertiary structure. The maintenance of catalytic activity of the mutants also indicates that the positive charge Arg^{37}-Arg^{38} is not so powerful to determine the overall conformation of the hTH1 molecule.

Next, we examined the efficiency of dopamine-mediated inhibition of the catalytic activities of these mutants. It is already known that the dopamine-mediated inhibition is caused by the formation of charge-transfer complex following the binding of dopamine to the active site iron located in the C-terminal two-thirds of the enzyme [31–36]. As was expected from our previous reports [5,6], our data in this research indicate that the positive charge of the amino acid residues at positions 37 and 38 is deeply involved in determining the efficacy of the dopamine-induced inhibition of the catalytic activity (Fig. 2). Again, the inhibitory effect of dopamine on the catalytic activity was pH-dependent for either wild-type or any mutants, which was coincided well with the previous report by Haavik et al. [37]. It is noteworthy that among the amino acid residues in the turn, Arg^{37}-Arg^{38} are the only ones that are electrically charged. It would thus be reasonable to speculate that the mutations of Arg^{37} and/or Arg^{38} to the neutral and/or negatively charged amino acids would cause some structural change in the turn. Although the overall shapes of the CD spectra were not different among wild-type and the mutants, the possibility of local structural changes induced by the mutations cannot be absolutely denied, because not huge but suggestive differences in the overall signal strength of the CD spectra were observed among them.

It has been suggested that the TH enzyme inactivated by the binding of dopamine can be reactivated by the phosphorylation of Ser residues in the N-terminus, which might expel dopamine from the TH molecule. The series of events should be one of the most important mechanisms to regulate the catalytic activity of the enzyme in vivo [4]. In spite of the presence of three phosphorylation sites in the N-terminus of hTH1, i.e. Ser^{19}, Ser^{31} and Ser^{40} [38], the fact that only Ser^{31} and Ser^{40} are readily phosphorylated to activate hTH1 in vitro has become a general consensus [7,8,39]. In this study, we indicated that Ser^{31} and Ser^{40} are located within the turn along with Arg^{37}-Arg^{38} (Fig. 1), which suggests that the Ser residues located near Arg^{37}-Arg^{38} can enhance the catalytic activity of the enzyme by their phosphorylation. Therefore, the positive charge intrinsic to Arg^{37}-Arg^{38} should largely contribute to maintain the characteristic of the turn and, as a result, control the efficiency of dopamine binding to (or dopamine expelling from) the enzyme. A theory that can explain the interrelationship among the positive charge of the specified amino acid residues, phosphorylation of Ser residues, and dopamine binding (or dopamine expelling) still remains to be constructed.

In conclusion, this is the first report to specify the importance of the positive charge of Arg^{37}-Arg^{38} in determining the efficiency of the dopamine inhibition from the perspective of the tertiary structure of the N-terminus 1–60 amino acid region of hTH1.

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