Purification and Characterization of Tyrosine Hydroxylase Histidine Mutant H331Q

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Contents

Abstract

A conserved histidine residue at position 331 of rat tyrosine hydroxylase was changed to glutamine by site-directed mutagenesis. The mutant enzyme, TOH-H331Q, was expressed in *E. coli* with a pET3b-based vector. The level of expression was far less than that of the wild-type enzyme; the best preparations were approximately 60% pure in TOH-H331Q, and represented only 0.01% by weight of the total protein. The H331Q mutant had markedly reduced activity, below the detection resolution of the assay. The mutant enzyme showed no response to free ferrous iron at concentrations up to 50-fold above saturating for the wild-type enzyme.

Acknowledgments

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INTRODUCTION

Tyrosine hydroxylase (TOH) catalyzes the conversion of the common amino acid tyrosine to dihydroxyphenylalanine (DOPA). This reaction is the rate-limiting step in the synthesis of catecholamines, a family of compounds which includes the adrenal hormones epinephrine and norepinephrine (adrenaline and noradrenaline), and the neurotransmitter dopamine (deficiencies in the production of which have been linked to Parkinson's disease). Additionally, the DOPA produced in the tyrosine hydroxylase step is the precursor for the biosynthesis of the pigment melanin (1).

The active enzyme is a tetramer that requires tetrahydrobiopterin and molecular oxygen as cosubstrates, together with non-heme ferrous iron, for activity. Together with dihydroxyphenylalanine, 4a-hydroxytetrahydrobiopterin is a product of the reaction (Figure 1). Product release is rapid, suggesting that a chemical step, rather than a protein conformational change, is rate-limiting (2). Further experiments with substrate and transition-state analogs indicate that tyrosine is not involved in the rate-limiting step. Rather, the key chemical step is probably the formation of an unidentified hydroxylating intermediate possibly 4a-peroxytetrahydrobiopterin (3).

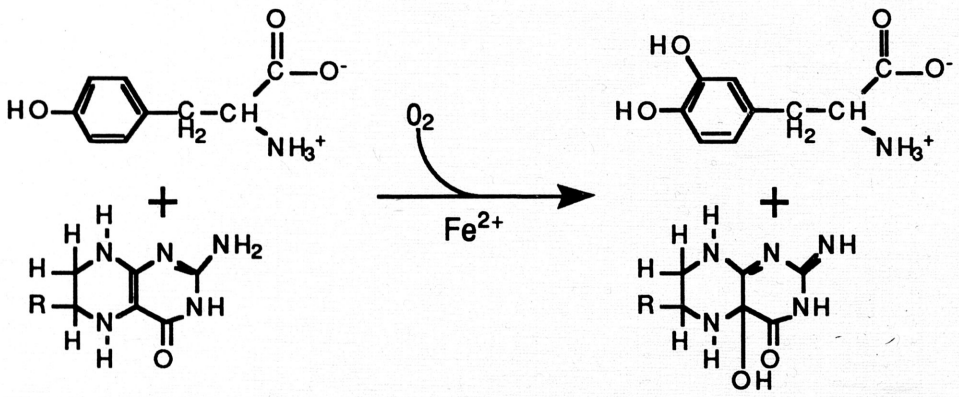

Figure 1: Scheme for the reaction catalyzed by tyrosine hydroxylase. Tyrosine is converted to dihydroxyphenylalanine (upper species) by the addition of molecular oxygen, with the concomitant oxidation of tetrahydrobiopterin to 4a-hydroxytetrahydrobiopterin (lower species). The enzyme requires non-heme ferrous iron for activity.

Like most rate-limiting enzymes, tyrosine hydroxylase is part of a complex physiological regulatory mechanism. Dopamine, epinephrine, and norepinephrine have been shown to be strong feedback inhibitors *in vitro*, and appear to coordinate with Fe^{3+} (4). Four phosphorylation sites have been identified. Phosphorylation of the rat enzyme at serine 40 by the cAMP-dependent protein kinase decreases its sensitivity to catecholamine inhibition (5). The effects of phosphorylation at the three other sites remain unknown.

Tryptophan hydroxylase and phenylalanine hydroxylase, two enzymes involved in similar reactions, are thought to have mechanisms closely related to that of tyrosine hydroxylase. Sequence comparisons of the enzymes reveal that the carboxy-terminal regions of all three enzymes are highly similar, while their amino terminal domains have little homology. The hypothesis that the C-terminal domain is the location of conserved catalytic function while the N-terminus is involved in regulation and substrate binding is supported by the result that the C-terminus alone has both tyrosine hydroxylase activity (6).

Tyrosine hydroxylase is also considered analogous to various lipoxygenases, which catalyze the addition of oxygen to double-bonds in polyunsaturated fatty acids in the biosynthesis of leukotrienes. The lipoxygenases too have a non-heme iron requirement. (7).

The role of iron in the active site of tyrosine hydroxylase is one of the most interesting questions posed by the enzyme, and is certainly its least understood attribute. Most purifications of tyrosine hydroxylase contain bound iron, and will stoichiometrically and reversibly bind divalent metal cations. The enzyme is activated 10- to 40-fold by the presence of Fe^{2+}, and binds but is inhibited by most other metals, including Fe^{3+}. The K_d for free Fe^{2+} is 0.15 μM (8). Metal binding is pH-dependent, suggesting that a proton-exchanging amino acid such as histidine is involved in the iron-

enzyme interaction (9). Few other details concerning the iron's role in catalysis are known.

Five conserved histidine residues have been identified in the lipoxygenase family. Nguyen *et al.* have used site-directed mutagenesis of human 5-lipoxygenase to demonstrate that two of these, H368 and H373, are required for activity and may be involved in the binding of iron (7). Balasubramian *et al.* have studied two conserved histidines (H138 and H143) in phenylalanine hydroxylase from *Cromobacterium violaceum* by site-directed mutagenesis and electron spin-echo envelope modulation. Their results indicate that while either one is sufficient for copper binding, both are required as coordination sites for the copper for full activity (10).

Tyrosine hydroxylase also has five conserved histidines, at positions 192, 247, 317, 331, and 336 in the rat enzyme (figure 2). H331 and H336 align with H138 and H143 of phenylalanine hydroxylase. Described here are the characteristics of the mutant protein in which H331 has been changed to glutamine, the δ-amino group of which has little affinity for iron, in order to probe the iron binding properties of the enzyme.

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                                                          40
MPTPS APSPQ PKGFR RAVSE QDAKQ AEAVT SPRFI GRRQS
                                                          80
LIEDA RKERE AAAAA AAAAV ASSEP GNPLE AVVFE ERDGN
                                                         120
AVLNL LFSLR GTKPS SLSRA VKVFE TFEAK IHHLE TRPAQ
                                                         160
RPLAG SPHLE YFVRF EVPSG DLAAL LSSVR RVSDD VRSAR
                                                         200
EDKVP WFPRK VSELD KCHHL VTKFD PDLDL DHPGF SDQVY
                                                         240
RQRRK LIAEI AFQYK HGEPI PHVEY TAEEI ATWKE VYVTL
                                                         280
KGLYA THACR EHLEG FQLLE RYCGY REDSI PQLED VSRFL
                                                         320
KERTG FQLRP VAGLL SARDF LASLA FRVFQ CTQYI RHASS
                                                         360
PMHSP EPDCC HELLG HVPML ADRTF AQFSQ DIGLA SLGAS
                                                         400
DEEIE KLSTV YWFTV EFGLC KQNGE LKAYG AGLLS SYGEL
                                                         440
LHSLS EEPEV RAFDP DTAAV QPYQD QTYQP VYFVS ESFND
                                                         480
AKDKL RNYAS RIQRP FSVKF DPYTL AIDVL DSPHT IQRSL
                        498
EGVQD ELHTL AHALS AIS
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Figure 2: Amino acid sequence of rat tyrosine hydroxylase. The conserved histidines are identified with boldface type.

EXPERIMENTAL PROCEDURES

Construction of Expression Vector pETOH1H331Q

The plasmid pTH6, containing the cDNA for rat tyrosine hydroxylase with a unique *Nde*I restriction site at the start codon and the gene for resistance to carbenicillin, was constructed as described previously (5). Site-directed mutagenesis (11) with the oligonucleotide 5'-ACTGC-TGCCA-AGAGC-TCTTG-GGACA-TG-3' (substituted bases are underlined) changed the CAT histidine codon at amino acid position 331 to a CAA glutamine codon and introduced a unique *Sst*I restriction site in order to simplify the screening procedure for mutants. The mutant TOH gene was excised from pTH6 by restriction with *Nde*I and *BamH*I and cloned into the plasmid pET3b of Studier *et al.* (12). The resulting plasmid, 6.4 kb in length and named pETOH1H331Q, had the TOH-H331Q gene downstream from the bacteriophage T7 RNA polymerase promoter, as well as carbenicillin resistance. This plasmid was transformed into competent *E. coli* strain BL21(DE3) pLysS, and permanent stocks were frozen at -80°C. These experiments were performed by Dr. Colette Daubner.

Expression of TOH-H331Q

E. coli strain BL21 lacks the *lon* and *ompT* proteases, making it suitable for overexpression of heterologous proteins. The DE3 lysogen is a bacteriophage λ derivative carrying the gene for the T7 RNA polymerase under the control of the *lac* operon. The phage DNA is inserted into the bacterial *int* gene; thus BL21(DE3) is stable (the lysogen will not excise without an external helper). The plasmid pLysS codes for T7 lysozyme, which inhibits T7 RNA polymerase at basal levels of transcription, and which aids in the lysis of cells during the protein purification. pLysS also confers resistance to chloramphenicol. Activation of the DE3 *lac* operon with lactose or a non-hydrolyzable analog such as isopropyl β-D-thioglucopyranoside (IPTG) causes transcription and translation of T7 RNA polymerase. The only promoter recognized by this highly active

enzyme in BL21(DE3) pLysS pETOH1H331Q cells is that for TOH-H331Q; this gene is thus transcribed at very high levels.

BL21(DE3) pLysS pETOH1H331Q cells were streaked for isolation from frozen stocks and grown for ≈12 hours at 37°C on Luria-Bertani medium agar plates containing 0.1 mg/mL carbenicillin and 0.05 mg/mL chloramphenicol (LB-carb-chlor). A single colony was then transferred to liquid LB-carb-chlor (approx. 12 mL per liter of final culture) and incubated at 37°C in a shaker flask at 300 rpm overnight. Ten mL of overnight culture were then transferred into 1.2 liters LB-carb-chlor and incubated at 37°C and 300 rpm. Expression was induced when the culture reached A_{600}= 0.6 - 0.7 by the addition of IPTG to a final concentration of 0.5 mM. Prior to induction, aliquots were spread onto LB, LB-carb, and LB-carb-IPTG plates. These plates were incubated overnight at 37° to screen for the ability of the culture to be induced. Cultures were grown for two to three hours following induction. The cells were then centrifuged at 8,600 x *g* for 20 min. The supernatant was discarded and the pellets were weighed and frozen overnight at -20°C.

Enzyme Purification

Cell pellets were thawed and resuspended in 6 mL homogenization buffer (50 mM Tris-Cl, 0.1 mM EDTA, 100 µg/mL phenylmethylsulfonyl fluoride, 1 µM leupeptin, 1 µM pepstatin, pH 7.0) per gram of cells. The cells were lysed by sonication. The sonicated cells were centrifuged at 16,000 x *g* for 40 minutes and the insoluble material was discarded. Ammonium sulfate (AS) was added in 5-10% increments up to 50% final saturation. After each addition, the solution was centrifuged at 16,000 x *g* for 30 minutes. Pellets were resuspended in heparin buffer (50 mM Tris-Cl, 0.1 mM EDTA, 10% glycerol, 1 µM leupeptin, 0.2 µM pepstatin, pH 7.0). Fractions were screened for TOH-H331Q by SDS-PAGE (13). Fractions displaying the appropriate 56 kD band were centrifuged at 17,500 x *g* for 20 minutes and the supernatants were loaded onto a heparin-

Sepharose column pre-equilibrated with heparin buffer. The column was washed with 50 mM Tris-Cl, 0.1 M KCl, 0.1 mM EDTA, 10% glycerol, 1 μM leupeptin, 0.2 μM pepstatin, pH 7.8, until $A_{280} \leq 0.02$. TOH-H331Q was then eluted with a 0.0 to 0.7 M KCl gradient in heparin buffer. Fractions were analyzed by SDS-PAGE, and those fractions containing TOH-H331Q were pooled and concentrated with a YM-30 membrane in an Amicon ultrafiltration cell. Protein solutions were kept at ≤ 4°C at all times. Protein concentrations were estimated by spectrophotometry ($\varepsilon_{280}^{1\%} = 10.4$ for tyrosine hydroxylase (14)) or Bradford dye-binding assays (15) with bovine serum albumin as standard.

Tyrosine Hydroxylase Activity Assays

The activity of tyrosine hydroxylase was determined by measurement of ^3H displaced to solvent from a 3,5-[^{3}H]tyrosine substrate. Assay conditions were as follows: 0.1 mM tyrosine, 0.05 M MES, pH 6.5, 0.075 mg/mL catalase, 1 μl/mL β-mercaptoethanol, and 0-500 μM ferrous ammonium sulfate in a final volume of 1 mL. Assays were conducted at a constant temperature of 32°C. Enzyme was pre-incubated in the assay mixture for 2-5 minutes prior to the initiation of the reaction by addition of 6-methyltetrahydropterin to a concentration of 0.5 mM. The reaction was quenched after 3 minutes by the addition of 50 μl glacial acetic acid. The reaction mixtures were loaded onto individual 0.5 x 2 cm Dowex 50 H^+ columns to remove unreacted labeled tyrosine. The tubes were rinsed with water which was also loaded onto the Dowex. The eluents were collected directly into vials and analyzed by liquid scintillation counting.

RESULTS

Expression and Purification of TOH-H331Q

Pre-induction log-phase cultures of BL21(DE3) pLysS pETOHIH331Q grew on LB and LB-carb agar plates to very high and similar levels. This result demonstrates that the cells were healthy at the time of induction and that almost all still contained the pETOH1H331Q plasmid. Additionally, growth of the identical culture was dramatically reduced on LB-carb-IPTG plates, usually to less than 10 colonies. This is the expected result when the cells induce successfully. T7 RNA polymerase is far more active than the native *E. coli* polymerase. When the pET expression system is functioning properly, the normal synthesis of RNA for cellular metabolism, controlled by the E. coli enzyme, is overrun by the transcription of the gene of interest. This results in the death of the cells, but usually not before a substantial quantity of the heterologous protein is produced. The lack of growth on IPTG plates indicates that heterologous mRNA for TOH-H331Q was over-produced.

However, examination of samples from various steps in the H331Q purification by SDS-PAGE did not reveal large amounts of the 56-kD product, which is clearly visible in comparable stages of the wild-type purification (Figure 3). Thus H331Q was either translated at a much lower level than wild-type, or destroyed by proteolysis.

Analysis by SDS-PAGE of samples taken at 45-minute intervals from an induced culture did not reveal any time-dependent loss of product due to proteolysis or another effect. Subsequent cultures were therefore grown for three hours in order to maximize yield.

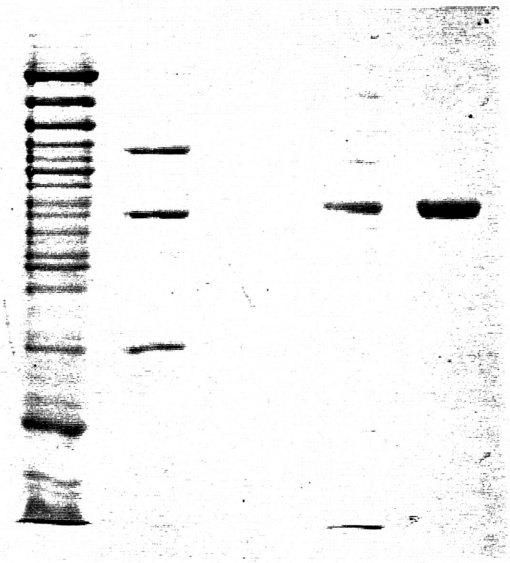

Figure 3: SDS-PAGE gel of tyrosine hydroxylase H331Q and wild-type samples. Lanes from left to right are: 40-45% AS pellet of H331Q, purified H331Q, 40-43% AS pellet of wild-type, and purified wild-type. Tyrosine hydroxylase migrates at 56 kD (center band in second lane). Note that the TOH band is just visible in the 45% AS pellet of the mutant enzyme, whereas it is the major component of the wild-type pellet.

The purification protocol for wild-type tyrosine hydroxylase involves ammonium sulfate fractionation followed by a heparin-Sepharose column. It was anticipated that the low levels of expression of the H331Q mutant would alter the concentrations of ammonium sulfate precipitation. Accordingly, solid ammonium sulfate was added to the soluble portion of the cell lysate in aliquots. The concentration of ammonium sulfate was increased in 5% increments from 30% to 50% saturation. After each aliquot the precipitating protein was collected by centrifugation. The individual fractions were analyzed by SDS-PAGE for enrichment of a protein with the correct molecular weight. The fractions containing the highest amount of this protein were then further purified by heparin-Sepharose chromatography, following the protocol developed for the wild-type enzyme. Individual column fractions were again analyzed by SDS-PAGE. Those which showed a protein with the correct molecular weight were then concentrated 5-10 fold by ultrafiltration for further analysis. Typical yields from this procedure were 190 μg from 25 g of cells, with an apparent purity of 60-75%.

Two contaminating proteins, migrating at 76 kD and 41 kD, typically co-eluted with the 56 kD TOH-H331Q (Figure 3). Gel filtration chromatography was used in an effort to separate the H331Q from these. One set of concentrated, pooled heparin-Sepharose fractions was run on a Superose-12 HR 10/30 FPLC column and the fractions were analyzed by SDS-PAGE. The separation achieved was incomplete; no highly pure TOH-H331Q was obtained, and some of the enzyme remained in fractions more concentrated with contaminant. This step was therefore not added to the standard purification protocol.

TOH-H331Q Activity

Tyrosine hydroxylase activity significantly different from blanks could not be detected under standard conditions (10 μM Fe^{2+} added) in any preparation of TOH-H331Q, in either the final purified form or in preliminary stages of the purification. Positive controls of wild type enzyme were run with each set of assays. The H331Q enzyme also did not have activity at free iron concentrations up to 500 μM.

Discussion

Although a high-resolution structure is not available, histidine 331 of tyrosine hydroxylase was identified as a putative iron ligand because

1) it is conserved in sequence comparisons between tyrosine, phenylalanine, and tryptophan hydroxylases from several species (6),
2) site-directed mutagenesis of the related human 5-lipoxygenase demonstrated that an analogous histidine is required for activity (7),
3) spectroscopic studies of tyrosine hydroxylase indicate that two or three histidines may be active site iron ligands (4), and
4) spectroscopy and site-directed mutagenesis of phenylalanine hydroxylase show that the equivalent histidine is an iron ligand required for activity (10).

In the work described here, no activity could detected under any conditions for this histidine to glutamine mutant. This establishes the activity of H331Q tyrosine as at least 1000-fold less than the wild-type enzyme. Such a result is expected if histidine 331 is indeed a ligand to the metal. However, it is also the expected result if this histidine has another role in catalysis or the structure of the protein. The possibility that the only effect of the mutation was on the enzyme's affinity for iron was examined by assaying the enzyme at an Fe^{2+} concentration 3300-fold that required for half-maximal binding to the wild-type enzyme. No activity was detected even at these levels. Because of the very low levels of expression of the mutant enzyme, it was not possible to obtain significant amounts of the pure protein for detailed physical characterization. More definitive conclusions will require mutagenesis of the other conserved histidines and the physical characterization of the iron binding of all five mutant proteins.

The reason for the low level of expression of TOH-H331Q remains a puzzling question. Examination of the growth of the expression cultures on selective media suggests that the T7 RNA polymerase is functional and that H331Q is therefore transcribed. A possible cause of the reduced expression is proteolysis and/or improper folding of the enzyme. The sequence change in the mutant does not introduce any new cleavage sites for common proteolytic agents; moreover, residue 331 is thought to be in the active site, in the core of the enzyme, where it would be inaccessible to proteases. The amino acid substitution also does not change the Chou-Fasman secondary structure predictions (16). These analyses, however, do not rule out the hypotheses that the tertiary structure is somehow disrupted. At present it is not possible to give an accurate explanation for this phenomenon.

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