Nitration and Inactivation of Tyrosine Hydroxylase by Peroxynitrite*

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Tyrosine hydroxylase (TH) is modified by nitration after exposure of mice to 1-methyl-4-phenyl-1,2,3,6-tetrahydroxyphenylpyridine. The temporal association of tyrosine nitration with inactivation of TH activity in vitro suggests that this covalent post-translational modification is responsible for the in vivo loss of TH function (Ara, J., Przedborski, S., Naini, A. B., Jackson-Lewis, V., Trifiletti, R. R., Horwitz, J., and Ischiropoulos, H. (1998) Proc. Natl. Acad. Sci. U. S. A. 95, 7659–7663). Recent data showed that cysteine oxidation rather than tyrosine nitration is responsible for TH inactivation after peroxynitrite exposure in vitro (Kahn, D. M., Aretha, C. W., and Geddes, T. J. (1999) J. Neurosci. 19, 10289–10294). However, re-examination of the reaction of peroxynitrite with purified TH failed to produce cysteine oxidation but resulted in a concentration-dependent increase in tyrosine nitration and inactivation. Cysteine oxidation is only observed after partial unfolding of the protein. Tyrosine residue 423 and to lesser extent tyrosine residues 428 and 432 are modified by nitration. Mutation of Tyr423 to Phe resulted in decreased nitration as compared with wild type protein without loss of activity. Stopped-flow experiments reveal a second order rate constant of (3.8 ± 0.9) × 10^4 M^-1 s^-1 at pH 7.4 and 25 °C for the reaction of peroxynitrite with TH. Collectively, the data indicate that peroxynitrite reacts with the metal center of the protein and results primarily in the nitration of tyrosine residue 423, which is responsible for the inactivation of TH.

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1 The abbreviations used are: TH, tyrosine hydroxylase; DAMBI, 4-(dimethylamino)phenylazophenyl-4’-maleimide; l-DOPA, l-dihydroxyphenylalanine; DTNB, 5,5’dithiobis(2-nitrobenzene); DTPA, diethylenetriaminepentaacetic acid; MPTP, 1-methyl-4-phenyl-1,2,3,6-tetrahydroxyphenylpyridine; NEM, N-ethylmaleimide; PD, Parkinson's disease; GST, glutathione S-transferase; MESS, 4-morpholineethanesulfonic acid; HPLC, high performance liquid chromatography; OPA, ortho-phenyldialdehyde; CD, circular dichroism; GdmHCl, guanidinium hydrochloride.

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alteration of methionine, tryptophan, or any other amino acid residues. Digestion and sequence analysis of peptides indicated that nitration of tyrosine 423 is the primary residue modified by peroxynitrite, which was further confirmed by a significant decrease in nitration of the Tyr123 → Phe mutant TH expressed in Escherichia coli. In addition, no loss of TH enzymatic activity was detected after peroxynitrite treatment of the Tyr123 → Phe mutant TH. Stopped flow experiments revealed reactivity with the ferrous iron in TH typical of metalloproteins reacting with peroxynitrite (15–17). The absence of other amino acid modifications at low peroxynitrite concentrations suggests that nitration of tyrosine 423 is responsible for the inactivation of TH by peroxynitrite.

MATERIALS AND METHODS

Purification and Activity Assay of TH—Recombinant tyrosine hydroxylase was isolated from BL21(DE3)pLyS8 E. coli expressing the full-length cDNA clone isolated from a rat pheochromocytoma library (18). E. coli were grown in LB broth in the presence of 0.1 mM FeSO4 to midlog phase and then induced with 1 mM isopropyl-1-thio-β-D-galactopyranoside for another 2 h. Addition of FeSO4 is essential to retain functional protein, because in the absence of ferrous iron salt, only 60% of the purified GTP cyclohydrolase I fusion protein was functional (19). The E. coli expressing the recombinant enzyme were pelleted in a 10-fold excess of 0.05 M Tris-HCl, pH 7.2 (w/v) and homogenized according to the method of Wang et al. (20). This mixture was sonicated with 30-s pulses at 20% power. After centrifugation, the supernatant was discarded and the pellet was resuspended in the same volume. The mixture was then sonicated with ten 30-s pulses. The supernatant was collected by centrifugation and removed. The pellet was resuspended and sonicated once again. The combined supernatants were precipitated with ammonium sulfate. The fraction between 30 and 40% saturation was purified further by column chromatography on heparin-Sepharose CL-6B. Tyrosine hydroxylase was eluted with a KCl gradient (0–0.7 M) in 0.05 M phosphate buffer, pH 6.5. The purity of peak fractions was evaluated by SDS-polyacrylamide gel electrophoresis and staining with Coomassie Blue and was found to be greater than 95%. Tyrosine hydroxylase was assayed by the release of [3H]tyrosine in the presence of catalase (21, 22). Approximately 5 μg of purified tyrosine hydroxylase was assayed in a volume of 30 μl containing the following additions: 50 mM MES, pH 5.5, 1 mM 6Rl-l-erythro-5,6,7,8-tetrahydrobiopterin (Alexis), 1500 units/ml catalase, 5 mM dithiothreitol, 0.10 mM tyrosine, 500,000 dpm of L-[3H]tyrosine (Montgomeryville, PA), and 1500 units/ml catalase. 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Table I

| Peroxynitrite | Cysteine residues per TH monomer | Tyrosine hydroxylase activity |
|--------------|---------------------------------|-----------------------------|
| μM           |                                 | % of control                |
| 0            | 6.8 ± 0.3                       | 100                         |
| 25           | 6.7 ± 0.4                       | 79 ± 11                     |
| 50           | 5.8 ± 1.2                       | 71 ± 11                     |
| 100          | 6.2 ± 0.8                       | 57 ± 6                      |
| 250          | 6.5 ± 0.6                       | 52 ± 10                     |
| 500          | 5.4 ± 1.1                       | 25 ± 11                     |

RESULTS

Lack of Cysteine Oxidation after Exposure of TH to Peroxynitrite—Purified recombinant rat TH was reacted with different concentrations of peroxynitrite, and fractions of the treated and control protein were analyzed for activity and for the concentration of reduced cysteine residues. Exposure to peroxynitrite resulted in a dose-dependent inactivation of TH activity (Table I). The concentration of cysteine residues in TH was determined by three different methods: ThioGlo-1, a maleimide-derivatized naphthopyranone, 5,5′-dithiobis(2-nitrobenzene) (DTNB), and 4-(dimethylamino)phenylazophenyl-4′-maleimide (DAMBI) labeling (24–26). Data in Table I show that in the unreacted protein seven cysteine residues are detected per TH monomer as predicted from the rat TH sequence. No evidence for cysteine oxidation was observed after treatment with up to 250 μM peroxynitrite whereas the TH enzymatic activity was already inhibited by more than 50%. With higher peroxynitrite concentration (500 μM), one cysteine residue per monomer is oxidized. The same results are obtained with DTNB and DAMBI labeling (not shown). These data suggest that TH activity was inhibited after exposure to peroxynitrite without evidence for oxidation of cysteine residues. Moreover, in addition to cysteine oxidation, the reaction of proteins in simple buffers with peroxynitrite has been shown to modify methionine, tryptophan, and tyrosine residues (10–14). Hydrolysis using methane sulfonic acid to analyze tryptophan and methionine as well as methionine sulfoxide failed to show any evidence for tryptophan and methionine oxidation (Table II). Complete amino acid analysis of peroxynitrite exposed TH, using gas phase HCl hydrolysis and PITC pre-column derivatization, did not detect any significant modification of any other amino acid residue (data not shown).

Peroxynitrite-induced Cysteine Oxidation in Partially Unfolded Tyrosine Hydroxylase—Tyrosine hydroxylase was partially unfolded with either 7 M urea or 6 M guanidine hydrochloride (GdmHCl) for 1 h at room temperature before peroxynitrite treatment. Exposure of the urea or GdmHCl partially unfolded protein to 500 μM peroxynitrite resulted in the oxidation of approximately three cysteine residues for 4.1 ± 1 and 3.2 ± 0.4 cysteine residues are detected in the reacted protein, respectively. Another fraction of the protein was analyzed by Western blotting with anti-TH antibodies, which revealed formation of SDS and heat-stable TH polymers (Fig. 1A). The polymerized TH was hydrolyzed, and the hydrolysates were monitored by fluorescence (λex = 283 nm and λem = 410 nm) to determine the presence of dityrosine as reported previously (33). The hydrolysates of TH treated with 6 M GdmHCl and reacted with 500 μM peroxynitrite contained 1 ± 0.3 μM dityrosine, indicating that one tyrosine residue in TH was oxidized to form dityrosine. The degree of cysteine and tyrosine oxidation in the unfolded protein was proportional to the peroxynitrite concentration (Fig. 1B). There was a significant degree of TH unfolding upon treatment with GdmHCl as revealed by the changes in the CD spectrum of the protein; the spectrum of the untreated protein and protein reacted with 1 M GdmHCl...
Three different tyrosine hydroxylase preparations reacted with peroxynitrite were analyzed for the formation of methionine sulfoxide (MetSO), methionine, and tryptophan content as described under “Materials and Methods.” The number indicates the number of residues measured per TH monomer. The reverse order experiment represents the addition of previously decomposed peroxynitrite to TH.

| MetSO/protein | Control | ONOO⁻ (100 μM) | Reverse order |
|---------------|---------|-----------------|---------------|
|               | 0.9     | 1.08            | 0.45          |

| Met/protein   | Control | ONOO⁻ (100 μM) | Reverse order |
|---------------|---------|-----------------|---------------|
|               | 4.1     | 4.8             | 4.1           |

| Trp/protein   | Control | ONOO⁻ (100 μM) | Reverse order |
|---------------|---------|-----------------|---------------|
|               | 2.7     | 2.4             | 2.8           |

**FIG. 1.** Polymerization and cysteine oxidation of partially unfolded TH. Tyrosine hydroxylase was treated with urea or guanidinium hydrochloride (GdmHCl) for 1 h at room temperature, centrifuged on a 10,000 molecular weight filter and diluted (18/19262) in 0.1 M phosphate buffer containing 0.1 mM DTPA, pH 7.4. A, the native and the partially unfolded TH was then reacted with 500 μM peroxynitrite and analyzed by Western blot analysis, using anti-TH antibodies. Lanes 1, 3, and 5 are control, urea-treated, and GdmHCl-treated protein; lanes 2, 4, and 6 are control, urea-treated, and GdmHCl-treated protein reacted with peroxynitrite. The inset table gives the cysteine content. B, tyrosine hydroxylase treated with 6 μM GdmHCl was reacted with different concentrations of peroxynitrite and the degree of protein cross-linking and cysteine oxidation was evaluated as in A.

Tyrosine Nitration following Exposure of TH to Peroxynitrite—Exposure of TH to a range of peroxynitrite concentrations induced a dose-dependent increase in the nitration of the protein, which correlated with the loss of enzymatic function (Fig. 3). The proportional loss of TH activity and nitration is similar to the data described in PC12 cells exposed to peroxynitrite (7). Assuming that one tyrosine residue is nitrated per TH molecule, then exposure of TH at 1, 2, and 3 mg/ml to 100 μM peroxynitrite resulted in the nitration of 59, 22, and 20% TH—

**Identification of the Nitrated Tyrosine Residues**—To identify the site of nitration, TH reacted with peroxynitrite was digested with either AspN or trypsin. The AspN-digested peptides were separated by reverse-phase HPLC, and two major peaks with absorbance at 275 and 365 nm eluted at retention times of 29.1 and 47.7 were collected and sequence analysis of the first 20 amino acids was performed. The following sequence, Asp-Thr-Ala-Ala-Val-Gln-Pro-Tyr-Gln (X represents an unknown amino acid) was obtained for the 29.1 min peak. This sequence corresponds to the expected AspN peptide residues 416–424 (Asp¹⁶-Thr-Ala-Ala-Val-Gln-Pro-Tyr-Gln), which contains a tyrosine residue in position 423. The peak with a retention time of 47.7 min contained two sequences, the major sequence (more than 90% abundance of amino acid residues) corresponding to peptide between residues 44 and 77, which does not contain any tyrosine residues. The minor sequence (less than 10%) was X-Gln-Thr-X-Gln-Pro-Val-X-Phe-Val-Ser-Glu-Ser-Phe, which corresponds residues 425–439 (Asp⁴₂⁵-Gln-Thr-Tyr-Gln-Pro-Val-Tyr-Phe-Val-Ser-Glu-Ser-Phe-Asn), which includes tyrosine residues 428 and 432. The site of nitration was also confirmed by digestion of nitrated TH with trypsin. The first 15-amino acid sequence of a peak eluted at 73 min of the tryptic digestion was Ala-Phe-Asp-Pro-Asp-Thr-Ala-Ala-Val-Gln-Pro-X-Gln (X represents an unknown amino acid), which corresponds to the expected peptide spanning residues 412 to 442 that includes tyrosine residues 423, 428, and 432. Collectively, these data suggest that the primary site of nitration is tyrosine residue 423 and to a lesser extent tyrosine residues 428 and 432. To further confirm the site of nitration, Tyr⁴²³ was mu-
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FIG. 4. Exposure of the partially purified wild type and Tyr423 → Phe mutant TH to peroxynitrite. A, wild type and Tyr423 → Phe mutant TH analysis by Western blot using a polyclonal anti-TH antibody. B, wild type and Tyr423 → Phe mutant TH analysis by Western blot using an anti-3-nitrotyrosine antibody, after treatment with a range of peroxynitrite concentrations. C, TH enzymatic activity (mean and standard deviation for three different preparations of TH) as a function of peroxynitrite concentration.

To purified TH, the sequence of the mutant TH (Tyr423 → Phe) expressed in E. coli was confirmed. Wild type and mutant proteins expressed in E. coli were partially purified and exposed to peroxynitrite under identical conditions. Fig. 4 shows that there was less nitration of the partially purified Tyr423 → Phe mutant as compared with the wild type protein under the same protein and peroxynitrite concentrations. However, the activity of the Tyr423 → Phe mutant TH was not affected by nitration as compared with the wild type protein. The activity of the Tyr423 → Phe mutant TH, similar to the wild type was decreased by 99% after heat inactivation or removal of the ferrous ion for the active site (Table III). In the semi-purified preparation the $K_m$ for tyrosine was 37.7 ± 2.1 μM for the wild type and 14.3 ± 2.6 μM for the Tyr423 → Phe mutant TH, which are similar to values reported for purified rat TH (34). The cysteine residues in the wild type and Tyr423 → Phe mutant TH appear to be equally reactive toward the alkylating agent N-ethylmaleimide (NEM). Treatment of the wild type and mutant proteins with NEM resulted in a significant inhibition of activity as shown in Table III. Moreover, NEM treatment also inhibited the activity of both the wild type and Tyr423 → Phe mutant TH proteins that had been reacted with peroxynitrite prior to NEM exposure (Table III). Collectively these data suggest that the cysteine residues were not modified by peroxynitrite exposure and that nitration of Tyr423 is the principal reason for the loss of enzymatic activity.

**DISCUSSION**

Tyrosine hydroxylase is the rate-limiting step in catecholamine synthesis, and thus the activity of this protein is critical for maintaining dopamine production. Inactivation of TH has been observed in early stages of PD as well as the mouse MPTP model of this disease (4). In the MPTP model of Parkinson’s disease (6) previous data revealed that TH is a protein specifically modified by nitration of tyrosine(s) residues. In addition, a temporal association between the number of TH molecules modified and loss of activity was observed (7). Amino acid analysis and fluorescence spectrometry of purified TH had failed to detect any other amino acid modification after nitration of the protein, and thus we proposed that tyrosine nitration is responsible for the inactivation of the protein (7).

However, a publication by Kuhn and co-workers (8) indicated that cysteine oxidation and not tyrosine nitration was responsible for the inactivation of purified TH by peroxynitrite in vitro. Although cysteine residues are well-recognized targets for peroxynitrite (9, 13, 26), this study failed to detect oxidation of cysteine residues after exposure of purified recombinant rat TH to peroxynitrite as determined by three independent methods. Cysteine oxidation was evident only after exposure of the protein to large excess of peroxynitrite or when the protein is partially unfolded (Table I and Figs. 1 and 2). It is possible that the protein used by Khun et al. (8) was partially unfolded during purification or removal of the glutathione S-transferase (GST) tag. It has been reported that nearly 40% of TH purified with the GST tag has no metal in the active site (19), and we have observed that metal free TH apoprotein readily aggregates after dialysis and during storage suggesting some degree of protein unfolding (not shown). Therefore, partial protein unfolding and exposure to high peroxynitrite concentration can account for the oxidation of cysteine residues reported previously (8). Consistent with the observation of Khan et al. (8) we also observed formation of SDS and heat-stable TH dimers after exposure to relative high peroxynitrite concentrations and in partially unfolded protein before and after exposure to peroxynitrite (Fig. 1). The dimers appear to be the result of cross-linking via oxidation of tyrosine residues, because ditryosine was detected in the unfolded and peroxynitrite treated TH. Dityrosine cross-linking has been reported for proteins

**TABLE III**

| Wild type TH | Tyr423 → Phe TH mutant |
|--------------|------------------------|
| % inhibition |                        |
| 500 μM peroxynitrite | 96 ± 3 | 96 ± 3 |
| 5 mM NEM | 93 ± 3 | 96 ± 3 |
| Heat-inactivated | 99.9 ± 0.1 | 99.8 ± 0.1 |
| Apo-protein | 99.8 ± 0.2 | 100 ± 0.1 |
with random secondary structure in solution (33) or globular proteins exposed to high peroxynitrite concentrations (36). Although protein cross-linking may be also responsible for the inactivation of TH, appreciable inactivation of the enzymatic activity is observed in the absence of protein dimers (Fig. 3) and other amino acid modifications suggesting that under these conditions tyrosine nitration is responsible for the loss of function (Tables I and II).

Peroxynitrite reactivity with proteins in simple buffers is determined by kinetic factors. The stopped-flow data suggest that the contribution of the ferrous iron in the reaction of peroxynitrite with TH is quite important, accounting for nearly 60% of the rate constant. The rate constant of the apoenzyme is typical of peroxynitrite reaction with protein amino acids (13) but precludes the existence of rapidly reacting cysteine residues, similar to those present in glyceraldehyde-3-phosphate dehydrogenase (37) and peroxiredoxins (38). Regarding the polypeptide chain, cysteine, methionine, and tryptophan are the primary amino acids that react with appreciable second order rate constants with peroxynitrite (13). In the case of TH, these amino acids were poorly oxidized after peroxynitrite exposure, similar to data reported previously for sarcoplasmic reticulum Ca-ATPase (39). Instead, at low peroxynitrite concentration, only tyrosine nitration was observed. At high peroxynitrite concentration, tyrosine nitration and oxidation were observed, which could be derived by the formation of hydroxyl and nitrogen dioxide radicals from peroxynitrite or by direct electrophilic substitution at the ortho position of the aromatic ring catalyzed by transition metals. However, the role of the ferrous iron of tyrosine hydroxylase in the nitration of the protein awaits further investigation.

The primary site of nitration was identified by digestion of the nitrated protein and peptide sequencing and further confirmed by mutational analysis (Fig. 4). The primary site of nitration was the tyrosine residue 423 and to a lesser extent tyrosine residues 428 and 432. Mutation of Tyr423 to Phe resulted in an appreciable decrease in tyrosine nitration and, more importantly, no loss of activity was observed after exposure to peroxynitrite. The specific activity of partially purified Tyr423 → Phe mutant was ~20–25% of the wild type protein suggesting that this tyrosine residue is critical for the enzymatic activity of the protein. However, similar to wild type protein, the mutant TH activity is completely lost upon heat inactivation and removal of the metal. Kinetic parameters such as the $K_{\text{TPR}}$ of the Tyr423 → Phe mutant were similar to the wild type, and the cysteine residues of both the wild type and Tyr423 → Phe mutant were sensitive to alkylation by NEM before or after treatment with peroxynitrite. Therefore, nitration of tyrosine 423 and not cysteine oxidation appears to be responsible for the inactivation of TH by peroxynitrite. However, it remains unclear how nitration of tyrosine residue 423 results in the inactivation of the protein. We speculate that it may relate to the critical positioning of this tyrosine residue near the active site of the protein. The active site of TH is located in the center of the catalytic domain (residues 188–456) and consists of a 17-Å deep cleft. The active site cleft is 30 Å long and 15 Å wide, and within the active site 10 Å below the enzyme surface His331, His336 and Glu376 residues bind ferrous iron needed for the catalysis of tyrosine hydroxylatation to l-DOPA (35). The entrance to the active site is guarded by two loops (residues 423–428 and 290–296), which come within 12 Å of each other. Proline residues in either side of the loop break the $\alpha$-helices, and Tyr423 starts the five-residue loop that ends with Tyr428. The aromatic ring of Tyr423 is oriented toward the opposite loop on the plane of the entrance to the active site whereas the aromatic rings of Tyr428 and Tyr432 are oriented away from the entrance to the active site. The narrow point entering the active site can be viewed as a size selection process by which only small substrates may enter into the active site. The addition of the bulky nitro (NO$_2$) in the ortho position of the tyrosine 423, but not the tyrosine residues 428 and 432, will narrow the distance between the two loops by more than 2.5 Å, which may be sufficient to prevent the entry of the substrate tyrosine in the active site of the protein.

Overall, the data reported herein are consistent with the view that tyrosine nitration is responsible for the inactivation of TH. It is now apparent that nitration and oxidation of proteins is a widespread event in the affected areas in the brain of PD patients (40–43) as well as in the mouse and baboon MPTP models of this disease (2, 7, 45). More importantly, specific proteins such as TH and α-synuclein that may play a key role in the pathogenesis of PD are targets for modification by nitrating agents (7, 42). Efforts to limit the formation of nitrating agents by limiting the production of nitric oxide and superoxide have been successful in protecting mice and baboons from MPTP-induced neuronal death (45–47). Recently, Pong et al. (48) showed that EUK 134, a superoxide dismutase and catalase mimetic, prevented nitration of TH in cultured dopaminergic neurons after 1-methyl-4-phenylpyridinium challenge. Therefore, development of therapeutic agents that can prevent formation of nitrating agents without interfering with normal neuronal function or compounds that will specifically remove nitrating species may protect proteins from inactivation and provide means of limiting neuronal injury in PD.

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