Tyrosine hydroxylase (TH), the initial and rate-limiting enzyme in the biosynthesis of the neurotransmitter dopamine, is inhibited by the sulfhydryl oxidant diamide in a concentration-dependent manner. The inhibitory effect of diamide on TH catalytic activity is enhanced significantly by GSH. Treatment of TH with diamide in the presence of $[^{35}\text{S}]\text{GSH}$ results in the incorporation of $^{35}\text{S}$ into the enzyme. The effect of diamide-GSH on TH activity is prevented by dithiothreitol (DTT), as is the binding of $[^{35}\text{S}]\text{GSH}$, indicating the formation of a disulfide linkage between GSH and TH protein cysteins. Loss of TH catalytic activity caused by diamide-GSH is partially recovered by DTT and glutaredoxin, whereas the disulfide linkage of GSH with TH is completely reversed by both. Treatment of intact PC12 cells with diamide results in a concentration-dependent inhibition of TH activity. Incubation of cells with $[^{35}\text{S}]\text{cysteine}$, to label cellular GSH prior to diamide treatment, followed by immunoprecipitation of TH shows that the loss of TH catalytic activity is associated with a DTT-reversible incorporation of $^{35}\text{S}$GSH into the enzyme. A combination of matrix-assisted laser desorption/ionization/mass spectrometry and liquid chromatography/tandem mass spectrometry was used to identify the sites of S-glutathionylation in TH. Six cysteines (177, 249, 263, 329, 330, and 380) of the seven cysteine residues in TH were confirmed as substrates for modification. Only Cys-311 was not S-glutathionylated. These results establish that TH activity is influenced in a reversible manner by S-glutathionylation and suggest that cellular GSH may regulate dopamine biosynthesis under conditions of oxidative stress or drug-induced toxicity.

Dopamine (DA)$^1$ neurons are targets for oxidative stress and drug-induced toxicity. Perhaps the most extreme example of such toxicity is that seen in Parkinson's Disease, a neurodegenerative disorder that results over time in the near-total destruction of the nigrostriatal dopamine system. Less extreme examples of toxicity to dopamine nerve endings are displayed by drugs of abuse from the amphetamine class, including methamphetamine and 3,4-methylenedioxymethamphetamine (1, 2). Drug-induced damage to DA neurons and the neuropathology associated with Parkinson's Disease are thought to be mediated, at least in part, by reactive oxygen and reactive nitrogen species (3–6).

The initial cellular response to oxidative stress is often a reduction in the levels of GSH and a corresponding increase of GSSG, the oxidized form of GSH (7–9). In the presence of various reactive species, GSH can form disulfide links with protein cysteine residues, a posttranslational modification referred to as S-glutathionylation (10–12). The enzymatic reduction of protein-GSH mixed disulfides is mediated by thioreductases, and these important enzymes are found in neurons (13), bacteria (14), and plants (15). The reversible modification of cysteines by GSH-disulfide linkage is thought to serve a protective function, masking critical protein sulfhydryls from more extensive or irreversible oxidation.

Thiolation-dethiolation reactions have been observed in many essential cellular systems including energy and respiratory function (16–18), signal transduction pathways (19–22), and transcriptional activation (23). Thiolation-dethiolation reactions in neurons are not as well understood as in nonneuronal cells, but evidence is emerging that these processes do occur after oxidative stress or in response to neurotoxicity. For example, metabolic inhibition in neurons leads to enhanced formation of protein-GSH mixed disulfides (24), and transgenic mice overexpressing thioltransferases in brain are protected from ischemia-induced neural damage (25). It also appears that cellular toxins and stressors can target protein sulfhydryl repair enzymes for inhibition (26, 27), leading to a shift in cellular redox balance favoring oxidation.

In view of the fact that cellular GSH levels and function are dramatically altered in Parkinson's Disease (28–30) and are, likewise, compromised by drugs known to damage DA nerves selectively including MPTP (31), 6-hydroxydopamine (32), and the amphetamines (33, 34), we considered the possibility that S-glutathionylation could influence DA neurochemistry. As there are no disulfides in the native structure of TH (35), each cysteine is subject to regulation by S-glutathionylation. We report presently that tyrosine hydroxylase (TH), the initial and rate-limiting enzyme in the synthesis of the neurotransmitter dopamine, is modified by S-glutathionylation and this post-

---

$^*$ This work was supported by NIDA, National Institutes of Health Grant DA10756 and by a Veterans Affairs Merit Award. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

$\square$ The on-line version of this article (available at http://www.jbc.org) contains a table showing assignment of all peaks from LC/MS/MS analysis and a figure showing the amino acid sequence of rat tyrosine hydroxylase.

$\square$ To whom correspondence should be addressed: Dept. of Psychiatry and Behavioral Neurosciences, Wayne State University School of Medicine, and the John D. Dingell Veterans Affairs Medical Center, Detroit, Michigan 48201

1 The abbreviations used are: DA, dopamine; ACN, acetonitrile; CHCA, α-cyano-4-hydroxycinnamic acid; CNBr, cyanogen bromide; DABMI, 4-dimethylaminophenylazophenyl-4'-maleimide; GfX, glutaredoxin; TH, tyrosine hydroxylase; HPLC, high pressure liquid chromatography; LC/MS/MS, liquid chromatography/tandem mass spectrometry; HRP, horse-

---

Dopamine Biosynthesis Is Regulated by S-Glutathionylation

POTENTIAL MECHANISM OF TYROSINE HYDROXYLASE INHIBITION DURING OXIDATIVE STRESS$^*$

Received for publication, September 4, 2002, and in revised form, September 27, 2002

Published, JBC Papers in Press, October 9, 2002, DOI 10.1074/jbc.M209042200

Chad R. Borges‡, Timothy Geddes§§, J. Throck Watson‡, and Donald M. Kuhn¶¶**

From the ¶Department of Biochemistry, Michigan State University, East Lansing, Michigan 48824, the $Department of Psychiatry and Behavioral Neurosciences and the ||Center for Molecular Medicine and Genetics, Wayne State University School of Medicine, and the ¶¶John D. Dingell Veterans Affairs Medical Center, Detroit, Michigan 48201

This paper is available on line at http://www.jbc.org
translational modification results in a reduction in TH catalytic function.

EXPERIMENTAL PROCEDURES

Materials—Tetrahydrobipterin was obtained from Dr. B. Schircks Laboratories (Jonsered, Switzerland). Transferrin, pGEX-4T vector, Salmon sperm DNA, a nickel affinity–selective column, pGEX-4T vector, and the glutathione S-transferase (GST) gene were purchased from Pharmacia. A nickel affinity–selective column from Nash Scientific was used to purify recombinant human GST. Nitrocellulose and polystyrene beads were purchased from Schleicher & Schuell. HPLC-grade solvents and other reagents were purchased from J. T. Baker (Phillipsburg, NJ) or Sigma (St. Louis, MO). Human recombinant thrombin was obtained from American Diagnostica. Antibody against TH was purchased from Roche Molecular Biochemicals, and horseradish peroxidase (HRP)-conjugated goat anti-mouse IgG was obtained from Cappel/Organon. Guanidine hydrochloride was obtained from Invitrogen (Carlsbad, CA). C18 ZipTips were purchased from Millipore (Bedford, MA). A Peptide CapTrap for LC/MS/MS was purchased from Michrom Biosciences (Auburn, CA). Acetonitrile (ACN) and trifluoroacetic acid were HPLC grade. All other reagents were of analytical grade from commercial sources.

Expression of Recombinant TH and Treatment with Diamide—TH was cloned by reverse-transcriptase PCR of rat brain RNA, and its sequence was verified by nucleotide sequencing. The full-length form of TH was ligated in frame into pGEX-4T2 and expressed as a GST fusion protein as previously described (36, 37). TH was cleaved from the GST fusion tag by treatment with thrombin. Native TH was purified to homogeneity from cultured PC12 cells as described by Kuhn and Billingsley (38). TH protein (3–5 μM, with respect to the 60-kDa monomer) was incubated with varying concentrations of diamide, GSH, or diamide + GSH in 50 mM potassium phosphate buffer, pH 7.5, containing 100 μM DPTA for 15 min at 30 °C. Samples were diluted 1:10 with potassium phosphate buffer, pH 7.5, and then TH catalytic activity was immediately assayed as described by Lerner et al. (39). Attempts to reverse the effects of diamide ± GSH on TH catalytic activity were carried out by adding DTT or GRX to dialyzed enzyme samples (2 h of dialysis against potassium phosphate buffer, pH 7.4) after treatment with diamide ± GSH, and incubations were continued for an additional 15–30 min at 30 °C before dilution and assay. Protein was measured using the method of Bradford (40).

Cell-free Measures of S-Glutathionylation of TH—Recombinant or purified PC12 TH was treated exactly as described above for the catalytic assays with the exception that GSH was replaced with [35S]GSH. The enzyme was subjected to non-reducing SDS-PAGE, and proteins were electroblotted to nitrocellulose. Blots were first exposed to film for autoradiography and were subsequently probed with a monoclonal antibody against TH. Immunoreactivity was visualized with the use of an HRP-conjugated goat anti-mouse antibody and enhanced chemiluminescence. In some instances, TH samples were treated with DTT or GRX after exposure to diamide + [35S]GSH, followed by SDS-PAGE and blotting to nitrocellulose.

Measurement of Diamide-induced TH Inactivation in Cultured PC12 Cells—PC12 cells were maintained in culture in Dulbecco’s modified Eagle’s medium containing 5% fetal calf and 10% inactivated horse serum in 5% CO2 incubators at 37 °C (38). PC12 cells were purified on a 15% sucrose cushion from a 15-min centrifugation step at 40,000 × g and harvested, and lysed by sonication. The supernatant fraction from a 15-min centrifugation step at 40,000 × g was split and treated with either 50 mM N-ethylmaleimide (to prevent scrambling of the [35S]GSH) or 25 mM DTT (to remove the [35S]GSH) prior to immunoprecipitation of TH. A polyclonal antibody raised against PC12 TH (38) was added to the supernatant fraction (1:1000 dilution of antiserum), and samples were incubated at 4 °C overnight. Immunoprecipitates were adsorbed to protein A-Sepharose at 4 °C for 2 h, washed 3× with 50 mM Tris-HCl buffer, pH 7.4, and eluted from beads with 10% SDS. Samples were exposed to non-reducing SDS-PAGE and electroblotting as described above. Blots were first exposed to film overnight at room temperature for autoradiography and were subsequently probed with a monoclonal antibody against TH. Immunoreactivity was visualized with the use of an HRP-conjugated goat anti-mouse antibody and enhanced chemiluminescence.

Chemical and Proteolytic Cleavage of TH—To determine sites of S-glutathionylation, TH was either subjected to cyanogen bromide cleavage followed by sample clean-up and analysis by MALDI/MS (Cys-329 and Cys-330 status determined in this manner) or cleaved with peptic and labeled with the chromophoric maleimide DABMI, followed by HPLC then analysis by MALDI/MS and LC/MS/MS. The proteolytic cleavage techniques described below were chosen because of their action at low pH values, which minimizes disulfide exchange. DPNH cleavage of TH was performed to isolate Cys-329 and Cys-330. Twenty micrograms of untreated TH, diamide-treated TH, and diamide + GSH-treated TH were purified after treatment by RP-HPLC. The HPLC system consisted of Waters Alliance 2695 pumps (controlled by a personal computer) in line with a Waters 2487 dual wavelength detector set at 314 nm. Initial chromatographic conditions were set at 15% solvent A (water containing 0.1% (v/v) trichloroacetic acid)/85% solvent B (ACN containing 0.1% (v/v) trichloroacetic acid) over a Vydac C18 (2.1 mm × 150 mm, 3-μm particle size, 300-A pores) column. Solvent composition was linearly ramped to 90% B over 60 min at 0.2 ml/min. Fractions corresponding to the whole protein were collected and dried in a vacuum system. One hundred twenty-six microliters of 6 μM guanidine HCl dissolved in 0.1 M HCl was added to each sample, followed by addition of 54 μl of 1 M CNBr in ACN. The reaction vessel was overlaid with nitrogen and allowed to react in the dark for 16 h. Excess CNBr and ACN were removed under vacuum, and samples were diluted to 360 μl with water. Half of each sample was removed and made 0.8% (v/v) in trichloroacetic acid. Samples were then cleaned up with a C18 ZipTip (Millipore) into 2 μl of 0.1 M acetic acid (90% volume) containing 1 M CNBr and 50% saturated with CHCA. The elution solution was spiked with 0.5 pmol each of bradykinin and insulin as internal standards bringing the total volume to 2.5 μl. 0.5 μl was then spotted onto a MALDI plate using the thin-layer spotting technique of Cadene and Chait (42).

Peptic cleavage was carried out to isolate all cysteines other than Cys-329 and Cys-330, the status of which were determined with CNBr cleavage followed by analysis with MALDI/MS/MS (above). Thirty-three μg of untreated TH, diamide-treated TH, and diamide + GSH-treated TH were purified by RP-HPLC as described above and dried under vacuum. Samples were then reconstituted in 165 μl of 6 M guanidine HCl in 10 mM potassium phosphate buffer, pH 7.4, and treated with 10 μg of 100 μM trypsin/ml in 100 mM HCl, and 6.6 μl of pepsin dissolved in 10 mM HCl at 500 ng/ml and allowed to digest for 16 h at 37 °C. One hundred sixty-five microliters of 6 μM guanidine HCl/0.1 M citrate buffer, pH 5, that had been saturated with ACN and DABMI were added to each sample and allowed to react for 1 h at room temperature. Because the salt concentration was too high to allow for immediate analysis of the digest by MALDI/MS or LC/MS/MS, a preliminary purification was carried out by RP-HPLC. The proteolytic peptides in the digest mixture were purified and separated by microbore RP-HPLC (Vydac C18 column: 1.0 mm × 150 mm, 5-μm particle size, 300-A pores, 50 μl/min) under a gradient held at 2% solvent B (defined above) for 12 min then ramped to 45% solvent B over 70 min. Half of each fraction was treated with all other fractions from this preliminary RP-HPLC run, then concentrated for analysis by LC/MS/MS. The remaining half of each fraction was dried under vacuum and reconstituted in 2 μl of the described MALDI matrix solution and spotted as described above.

c's modified Eagle’s medium lacking sulfur-containing amino acids and containing 10% dialyzed serum for 18 h at 37 °C. The protein synthesis inhibitor cycloheximide (50 μg/ml) was added to cells and after 1 h, [35S]cysteine (30 μCi/ml) was added, and incubations continued in the presence of cycloheximide for another 4 h at 37 °C. Cells were washed 3× with Krebs-Ringer phosphate buffer to remove unincorporated isotope, and exposed to 10 μM diamide for 15 min at 37 °C as described above. Cells were next washed 3× with Krebs-Ringer phosphate buffer, harvested, and lysed by sonication. The supernatant fraction from a 15-min centrifugation step at 40,000 × g was split and treated with either 50 mM N-ethylmaleimide (to prevent scrambling of the [35S]GSH) or 25 mM DTT (to remove the [35S]GSH) prior to immunoprecipitation of TH. A polyclonal antibody raised against PC12 TH (38) was added to the supernatant fraction (1:1000 dilution of antiserum), and samples were incubated at 4 °C overnight. Immunoprecipitates were adsorbed to protein A-Sepharose at 4 °C for 2 h, washed 3× with 50 mM Tris-HCl buffer, pH 7.4, and eluted from beads with 10% SDS. Samples were exposed to non-reducing SDS-PAGE and electroblotting as described above. Blots were first exposed to film overnight at room temperature for autoradiography and were subsequently probed with a monoclonal antibody against TH. Immunoreactivity was visualized with the use of an HRP-conjugated goat anti-mouse antibody and enhanced chemiluminescence.
Regulation of Tyrosine Hydroxylase by S-Glutathionylation

![Graph](Image)

Fig. 1. Inactivation of TH by diamide and its enhancement by GSH. Recombinant TH (10 μM) was treated for 15 min at 30 °C with the indicated concentrations of diamide in the absence (○) or presence (●) of GSH (1 mM), and the amount of TH activity remaining after treatment was determined. The results are expressed as % control TH activity (GSH alone) and represent the means ± S.E. of five experiments run in duplicate. The effects of diamide and diamide + GSH on TH activity were significant (p < 0.05 by ANOVA) as was the enhancement of diamide-induced inactivation of TH by GSH (p < 0.05 by ANOVA).

Determination of S-Glutathionylation sites in TH by Mass Spectrometry—A Voyager DE-STR MALDI-TOF MS (PE Biosystems Inc., Framingham, MA) equipped with a 337-nm laser was used with external standards of bradykinin and bovine pancreatic insulin. LC/MS/MS analysis was carried out on a Waters CapLC system connected to a ThermoFinnigan LCQ Deca ion trap. Duplicates from each sample were injected onto a Peptide CapTrap (Michrom Bioresources), washed for 6 min at 20 μl/min with water/ACN (98/2, v/v) containing 0.1% (v/v) formic acid, separated on a 75-μm × 5-cm ProteoPep C18 PicoFrit column, and analyzed by LC/MS and LC/MS/MS.

Analysis of Mass Spectrometry Data—DABMI-labeled peptides produce a unique set of peaks when analyzed by MALDI/MS due to prompt fragmentation reactions that occur when the laser strikes the sample (e.g., see Fig. 7). This set of peaks allows for easy recognition of DABMI-labeled (i.e. originally free cysteine-containing) peptides in the MALDI mass spectrum without the use of MS/MS and without knowledge of the peptide sequence. A more detailed report on the chemistry and application of DABMI as a tool for protein science will be prepared separately. A mass mapping approach was employed to elucidate the structure of putative cysteine-containing peptides that were recognized through DABMI labeling. Based on the mass of the DABMI-labeled peptides, masses of the corresponding glutathionylated peptides were calculated; hits for these calculated masses (via a search using TurboSequest) were confirmed after analysis by LC/MS/MS. Actual peak assignments in MS/MS spectra were made manually. For a spectrum to be considered a valid hit for a glutathionylated peptide, all peaks with a relative intensity greater than 10% had to be assigned; as it turned out, ~90% + of all peaks greater than 5% relative intensity were assigned. Assignments also were required to be consistent with fragments expected from a low-energy collision-induced dissociation ion trap mass spectrometer. Several candidate MS/MS spectra were rejected due to failure to meet the above criteria.

RESULTS

TH activity is reduced by the highly specific thiol oxidant diamide. Fig. 1 shows that increasing concentrations of diamide cause a slight inhibition of TH between concentrations of 0.1–0.5 mM, but concentrations of 1–5 mM result in ~30–40% inhibition of catalytic activity. The addition of a constant concentration of GSH (1 mM) to incubations with varying concentrations of diamide substantially enhances the inhibition of TH. For example, GSH increased the inhibition of TH to 34% of control in the presence of 1 mM diamide, whereas without GSH, diamide reduced TH activity to only 70% of control. Treatment of TH with GSH alone did not have an effect on enzyme activity in concentrations up to 10 mM (data not shown). The effect of diamide, as well as the combination of GSH + diamide, on TH activity was statistically significant (p < 0.05 for each condition, ANOVA). The GSH-induced enhancement of the diamide effect on TH was also statistically significant (p < 0.05, ANOVA). GSH-induced enhancement of the diamide effect on the catalytic activity of TH purified from PC12 cells was the same as observed with recombinant TH (data not shown).

The effect of the sulfhydryl-reducing agent DTT on TH inhibition caused by diamide + GSH was tested, and the results are presented in Fig. 2A. When DTT (1 mM) was added to incubations prior to GSH + diamide, the inhibition of TH was completely prevented. DTT also prevents the inhibition of TH caused by diamide alone (data not shown). However, attempts to reverse inhibition of TH caused by GSH + diamide were somewhat less effective. Fig. 2B shows that the addition of DTT (10 mM) to TH after exposure to GSH + diamide partially restores TH catalytic activity. DTT led to the recovery of ~50% of the lost activity, an effect that was statistically significant (p < 0.05, Bonferroni’s test). Neither higher concentrations nor longer incubation times increased the extent to which the inhibition of TH could be reversed by DTT (data not shown). GRx was also tested for the ability to reverse the inhibition of TH caused by diamide + GSH, and the results are presented in Fig. 2B. GRx (1 μM), in the presence of GSH (0.5 mM), caused a significant recovery of TH activity. The recovery of activity caused by GRx was slightly greater in magnitude than that of DTT, reaching ~60% of control. The effect of GRx + GSH was
The sites of S-glutathionylation in TH were determined by MALDI/MS and LC/MS/MS. Because two of the cysteine residues in TH are juxtaposed (i.e. Cys-329 and Cys-330), CNBr proved to be the most effective cleavage technique to obtain these residues in a single peptide. A peak for the expected cleavage product \( H_2N-H-SPEDPCHELLGHVPMm \), where \( M_h \) indicates a homoserine lactone residue, was readily found in the MALDI mass spectra of both the untreated and diamide-treated TH (Fig. 6). Interestingly, the \( m/z \) value found corresponded to the expected cleavage product containing an intramolecular disulfide bond (calculated monoisotopic mass of 1850.8 Da for [M+H]+). This disulfide bond is not a feature of wild type TH and is an artifact of the peptide preparation conditions. Trace C of Fig. 6 is from diamide + GSH-treated TH. The amount of unglutathionylated peptide is dramatically decreased, and there is a small peak that corresponds to a monoglutathionylated peptide (calculated monoisotopic mass of 2157.9 Da for [M+H]+). Most significantly, there is an intense peak that corresponds to a diglutathionylated peptide (calculated average mass of 2464.8 Da for [M+H]+), indicating that both Cys-329 and Cys-330 are glutathionylated. The peaks at \( m/z \) 1850.5 and \( m/z \) 2158.0 may represent either incompletely glutathionylated peptides or prompt fragmentation products produced when the laser strikes the sample (43).
Peptide cleavage and DABMI labeling were used in combination with LC/MS/MS to identify remaining S-glutathionylated sites in TH. A total of 32 different DABMI-labeled peptides were found by MALDI/MS in the HPLC fractions of pepsin-cleaved and DABMI-labeled untreated TH. This means that on average, each of the seven cysteines in the TH monomer was distributed among four to five different peptides. A representative MALDI mass spectrum of a set of peaks corresponding to a DABMI-labeled peptide is shown in Fig. 7. In this case, for reasons explained in the “Analysis of Mass Spectrometry Data” above, the ions representing the DABMI-labeled peptide could correspond to five possible cysteine-containing peptides, each containing Cys-177, Cys-263, or Cys-311. Once mass spectral peaks for DABMI-labeled peptides were recognized based on their signature pattern, the experimentally determined m/z value of the [M+H]+ ion was theoretically shifted by −15.1 units to obtain the expected m/z value for the corresponding peptide if it were glutathionylated instead of DABMI-labeled. These shifted m/z values were searched for in the mass spectra of the diamide + GSH-treated TH sample, but peaks were disregarded if also present in a control sample. A few glutathionylated peptides were found in this manner, but it was not possible to assign a cysteine in all cases (as in the above case involving Cys-177) using MALDI/MS alone because the proteolytic scheme used here could generate several cysteine-containing fragments having a calculated mass in good agreement with the mass spectral data. Analysis of another aliquot of the digest by LC/MS/MS was used to confirm the identity of the glutathionylated peptide.

Fig. 8 shows an MS/MS spectrum that confirms glutathionylation of Cys-177. The spectrum is of the same peptide (in this case glutathionylated, not DABMI-labeled) that produced the MALDI/MS spectrum in Fig. 7, and it was found in both the “triple play” and the “Big 3” analyzed injections, but not in the untreated or diamide-treated TH samples. Peak assignments from other MS/MS spectra were used to identify Cys-177, Cys-249, Cys-263, and Cys-380 as being glutathionylated in the same way as described above for the peptide containing Cys-177. At least one of the MS/MS spectra supporting glutathionylation for each of these cysteines was produced from a protonated molecule also observed by MALDI/MS. These MS/MS data are included in Table I of the Supplemental Data section found at http://www.jbc.org. No evidence was found for glutathionylation of Cys-311. MALDI/MS data from diamide + GSH-treated TH (not shown) indicated that at least a significant portion of Cys-311 remains free following diamide and glutathione treatment.

**DISCUSSION**

TH is an oxidatively labile enzyme whose level of activity is determined, in part, by the redox status of its cysteine sulfhydryls. For instance, the sulfhydryl oxidants peroxynitrite and tetranitromethane (37) or catechol-quinones (44) reduce TH activity to an extent that is related to cysteine modification. It has also been proposed that the reductions in TH function that are seen under conditions that damage DA neurons reflect an attack on the protein by reactive oxygen or nitrogen species (45). The present results confirm and extend these findings and add a new dimension to the understanding of how dopamine neurons and TH may respond to oxidative stress. TH is slightly inhibited by the specific thiol oxidant diamide (46) and this inhibition of enzyme activity is significantly enhanced by GSH. The characteristics of the inhibition of TH by diamide + GSH are entirely consistent with other studies that have established that proteins can be modified by disulfide linkages with low molecular weight thiols such as GSH (10–12). First, the inhibition of TH activity caused by GSH + diamide can be prevented by DTT. Second, the inhibition of TH activity is partially reversible by DTT and GRx. Third, GSH forms a DTT and GRx-reversible disulfide link with TH cysteinyl residues as evidenced by incorporation of [35S]GSH into the protein under oxidizing conditions. Taken together, these results establish that TH can be modified by S-glutathionylation.

It is often observed that alterations in the function of proteins that have been modified by S-glutathionylation can be reversed by thiol reducing agents (19, 22). However, we were unable to reverse completely the catalytic effects of S-glutathionylation of TH even though reducing agents cleaved the disulfide linkage between [35S]GSH and TH cysteinyl residues. Higher concentrations of reducing agents, variations in the temperature of the reversal incubations, or increases in the time of the reversal reactions did not increase the recovery of activity to more than −50%. This result could indicate that TH has been modified under the present oxidizing conditions at sites other than cysteine residues, and we cannot rule out this unlikely possibility. It may also be the case that at elevated...
temperatures in the presence of oxygen some TH sulphhydrils are oxidized beyond disulfides to a point at which reduction is more difficult. By analogy, the inhibition of TH caused by peroxynitrite is not reversible and is accompanied by the oxidation of cysteines beyond sulfenic acid (37). For some proteins, on the other hand, complete recovery of function can be seen after only partial deglutathionylation with DTT or GRx (47, 48). These findings indicate that the reactivity of certain cysteine residues to glutathionylation is determined by numerous factors and that the impact of glutathionylation is determined by the contribution of the modified cysteine residue to protein function. For example, glutathionylation of Cys-199 in protein kinase A inhibits its activity, whereas the same modification at Cys-343 has no effect (47). We did observe a lag phase in the inactivation of TH by diamide when GSH was present (Fig. 1), suggesting the possibility that glutathionylation involved one or a few less reactive cysteines, and this possibility is currently under investigation.

The in vitro results demonstrating S-glutathionylation of TH were extended to a cellular model. PC12 cells were treated with diamide, and it was observed that TH activity was reduced in a concentration-dependent manner. Immunoprecipitation of TH after labeling of cellular GSH with [35S]cysteine revealed that diamide treatment resulted in the incorporation of an 35S label into TH in a DTT-reversible manner. This result does not simply reflect synthesis of new TH protein because translation...
Regulation of Tyrosine Hydroxylase by S-Glutathionylation

had been inhibited with cycloheximide and TH was not labeled with [35S]GSH in the absence of diamide. These studies in a cell culture model substantiate the in vitro studies with purified TH and suggest that cellular TH activity can be modulated by S-glutathionylation under conditions of oxidative stress (i.e. in the presence of diamide).

A combination of MALDI/MS and LC/MS/MS was used to search for sites of S-glutathionylation in TH. CNBr and peptic cleavage were used at an acidic pH to minimize disulfide formation during the preparation and isolation of peptide fragments. TH has seven cysteine residues per 60-kDa monomer (49), and all appear to be free in the native enzyme (35). CNBr cleavage of TH produced the peptide H$_2$N-HISPEPDCCHELL-GHVPM$_x$, where M$_x$ indicates a homoserine lactone residue, containing Cys-329 and Cys-330. MALDI/MS detected a mass shift indicative of deglutathionylation of the peptide fragment and allowing unequivocal assignment of Cys-329 and Cys-330 as sites of S-glutathionylation. The remaining CNBr fragments of TH were too large for accurate characterization by MALDI/MS, so we resorted to peptic cleavage and LC/MS/MS to determine the status of the remaining cysteine residues. A total of 32 cysteine-containing peptides (as recognized by DABMI labeling) were isolated after peptic cleavage, indicating that on average, each cysteine in TH was found in four to five peptides. MALDI/MS was used to identify those peptides containing cysteines by virtue of the mass spectrum signature associated with DABMI, but in general could not be used to assign specific cysteine residues. Proceeding to LC/MS/MS analysis, it was possible to identify and characterize all remaining cysteines in TH with the exception of Cys-329 and Cys-330 (assigned with MALDI/MS, above). It was observed that cysteines 177, 249, 263, and 380 were S-glutathionylated. Only Cys-311 was not modified and remained substantially free after treatment with S-glutathionylating conditions. Based on these results, it does not appear that any single cysteine residue in TH is critical for catalytic activity. The conditions that resulted in S-glutathionylation of six of the seven cysteines in TH lowered catalytic activity by about 70–80%. We have also found that the modification of cysteines in TH by catechol-quinones follows a similar pattern in that inhibition of catalytic activity is highly correlated with the number of cysteines that are modified (44).

GSH is an important member of the cellular antioxidant defense system, and it has been estimated that its cellular concentration can be as high as 10 mM (50). It is somewhat perplexing, therefore, how oxidative or nitrosative stress can lead to cellular toxicity or death. Only recently has it become evident that GSH can form disulfide links with protein cysteinyI residues during oxidative stress. This posttranslational modification is referred to as S-thiolation or, more appropriately, S-glutathionylation (10–12). Many cell types respond to oxidizing conditions with a change in the balance between GSH and its oxidized form GSSG. In some instances, S-glutathionylation serves a protective function by masking critical sulfhydryl groups in proteins from more extensive and irreversible oxidation (e.g. to sulfonic or sulfonic acid). The number of proteins known to be modified by S-glutathionylation remains small, yet precedence does exist for S-glutathionylation-induced loss of function. For example, thioredoxin itself is now known to be inhibited by S-glutathionylation (51). Protein kinases A (47) and C-α (22) are inhibited by S-glutathionylation as is c-Jun DNA binding activity (23). Cellular protein ubiquitinylated (20) and the phosphatase activity of carboxy anhydrase III (16) are also subject to redox regulation by disulfide linkage with GSH. It now appears that TH can be added to the list of proteins whose function is modulated by S-glutathionylation under conditions of oxidative stress.

The finding that TH is subject to regulation by S-glutathionylation may be relevant to conditions known to stress or damage dopamine neurons. These include the neurodegeneration associated with Parkinson’s Disease and dopamine neuronal toxins (e.g. 6-OHDA and MPTP (1-methyl-4-phenyl-1,2,3,6-tetrahydro-pyridine)), as extreme examples, and the damage caused by methamphetamine, as a more moderate example. The damage to dopamine neurons associated with these conditions includes reductions in TH catalytic function that has been attributed, at least in part, to oxidative stress (1, 45). They also involve alterations in GSH levels and function (28, 30–33). It is generally accepted that reactive oxygen species (e.g. hydroxyl radical, superoxide) or reactive nitrogen species (e.g. nitric oxide, peroxynitrite), which are produced under conditions of oxidative stress, play direct roles in dopamine cell damage. However, it may well be the case that oxidative stress within dopamine neurons leads to TH S-glutathionylation via the reaction of oxidizing species with GSH, and this mechanism reduces TH activity.

It is not surprising that TH is inhibited by the specific thiol oxidant diamide in view of the ability of many sulfhydryl oxidants to inactivate TH catalytic activity (37, 44). Interestingly, unless the molar ratio of GSH to diamide is greater than 5, GSH enhances diamide-induced inhibition of TH instead of providing protection. This is reasonable because sulfhydryl groups within TH would remain as mixed disulfides with glutathione unless the molar ratio of free GSH was high enough to predominately form GSSG disulfides instead of GSSC disulfides (where C represents a cysteine within TH) through disulfide exchange. Diamide alone, however, may simply react reversibly with free sulfhydryl-containing cysteines within TH under the experimental conditions and upon dilution may leave most cysteines within TH unaffected. (In general, cysteines are not “irreversibly” oxidized by diamide until GSH reacts with the cysteine-diamide complex.) In any case, the redox status of TH cysteine sulfhydryls is an important determinant of its level of function, and it is interesting to speculate that oxidative stress within DA neurons leads to a GSH-mediated alteration in TH catalysis. While glutathionylation may protect some proteins from damage, it is possible that TH cannot tolerate even relatively mild modification of its cysteines through disulfide linkage with GSH. Furthermore, it is plausible that a surge of oxidative stress could result in a reduction in TH function that is not prevented by the high cellular concentrations of GSH but is actually mediated by it. These possibilities are currently under investigation.

Acknowledgment—We thank Dr. John Mieyal for expert advice on GRx and its use in dethiolation experiments.

REFERENCES

1. Gibb, J. W., Johnson, M., Elayan, I., Lim, H. K., Matsuda, L., and Hanson, G. R. (1997) NIDA Res. Monogr. 173, 128–145
2. Cadet, J. L., and Braunschweig, C. (1996) Neurochem. Int. 22, 117–131
3. Good, P. F., Hsu, A., Werner, P., Perl, D. P., and Olanow, C. W. (1998) J. Neuropathol. Exp. Neurol. 57, 338–342
4. Gutteridge, J. M. (1994) Ann. N. Y. Acad. Sci. 738, 201–213
5. Glanow, C. W., and Tatton, W. G. (1999) Annu. Rev. Neurosci. 22, 123–144
6. Simonian, N. A., and Coyle, J. T. (1996) Annu. Rev. Pharmacol. Toxicol. 36, 83–106
7. Cotgreave, I. A., and Gerdes, R. G. (1996) Biochem. Biophys. Res. Commun. 224, 1–9
8. Dringen, R., Gutterer, J. M., and Hirrlinger, J. (2000) Eur. J. Biochem. 275, 4912–4916

* C. R. Borges and J. T. Watson, unpublished observations.

S. A. Rovinsky, personal communication.
