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Inactivation of Dopamine β-Hydroxylase by *p*-Cresol: Isolation and Characterization of Covalently Modified Active Site Peptides

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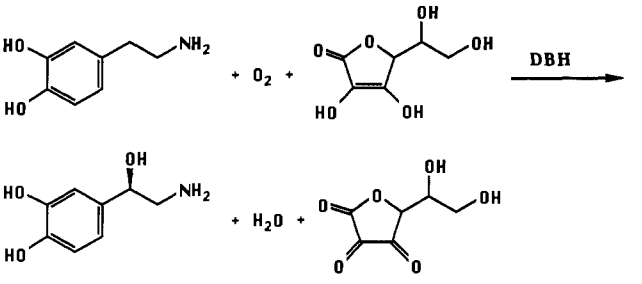
ABSTRACT: Recently, *p*-cresol has been shown to be a mechanism-based inhibitor of dopamine β-hydroxylase (DBH; EC 1.14.17.1) [Goodhart, P. J., DeWolf, W. E., Jr., & Kruse, L. I. (1987) *Biochemistry 26*, 2576–2583]. This inactivation was suggested to result from alkylation of an active site residue by an aberrant 4-hydroxybenzyl radical intermediate. In support of this hypothesis, we report here the isolation and characterization of two modified tryptic peptides from DBH inactivated by *p*-cresol. Using a combination of automated Edman sequencing, mass spectroscopy (MS), and tandem MS, we have determined the sequence of the putative active site peptides, identified the site of attachment of *p*-cresol, and defined the chemical nature of the adduct formed. Both modified peptides are the same primary sequence: Ala-Pro-Asp-Val-Leu-Ile-Pro-Gly-Gln-Gln-Thr-Thr-Tyc-Trp-Cys-Tyr-Val-Thr-Glu-Leu-Pro-Asp-Gly-Phe-Pro-Arg, where Tyc is an amino acid residue with the in-chain mass of a cresol–Tyr adduct (106 + 163 Da). Gas-phase deuterium exchange studies (employing N^2H_3-DCI MS) of the isolated phenylthiohydantoin (Pth) derivatives of modified residue 13 demonstrate that *p*-cresol forms two chemically distinct covalent adducts and support the hypothesis that a (4-hydroxyphenyl)methyl radical is generated during catalysis. Rearrangement to a (4-methylphenyl)oxy radical may also occur prior to inactivation.

Dopamine β-hydroxylase (DBH;[1] EC 1.14.17.1) is a copper-containing mixed-function oxidase that catalyzes the hydroxylation of dopamine to norepinephrine (Scheme I; Levin et al., 1960; Skotland & Ljones, 1979; Rosenberg & Lovenberg, 1980; Villafranca, 1981; Ljones & Skotland, 1984). Despite its key physiological role in the biosynthesis of neurotransmitters and its absolute stereochemical specificity for the *pro-R* hydrogen (Battersby et al., 1976), DBH displays a remarkable lack of specificity toward organic substrates. In addition to hydroxylating a number of substituted phenethylamines and phenylpropylamines (Creveling et al., 1962), DBH has been shown to catalyze sulfoxidation of phenyl thioethers (May & Phillips, 1980; May et al., 1981), epoxidation of styrenes, and N-dealkylation of methylamines (Padgette et al., 1985). In recent years, the indiscriminate nature of DBH has led to numerous reports of mechanism-based inhibitors; for an extensive review, see Fitzpatrick and Villafranca (1987). Various speculative chemical mechanisms have been advanced to account for the observed inactivation. These implicitly assume the involvement of the latent elec-

Scheme I



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[1] Abbreviations: Da, dalton(s); DBH, dopamine β-hydroxylase; DCI, desorptive chemical ionization; DPM_T, total disintegrations per minute; FAB, fast atom bombardment; FABMS, fast atom bombardment mass spectroscopy; HPLC, high-performance liquid chromatography; MS, mass spectroscopy; NEMAc, *N*-ethylmorpholineacetate; Pth, phenylthiohydantoin; SDS–PAGE, sodium dodecyl sulfate–polyacrylamide gel electrophoresis; TFA, trifluoroacetic acid; TPCK, *N*-tosyl-L-phenylalanine chloromethyl ketone.

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trophilic groups generally present in the inactivator. However, as has been correctly pointed out (Fitzpatrick & Villafranca, 1987), definitive characterization of the mechanism by which a particular compound inactivates is predicated upon determination of the chemical structure of the adduct formed in the inactivated enzyme. Despite the plethora of mechanism-based inhibitors reported for DBH, in only a few cases has alkylation of the enzyme actually been demonstrated (Fitzpatrick & Villafranca, 1986; Colombo et al., 1984; Kandategе & May, 1987), and in no case has the structure of the adduct been determined.

We have recently reported that *p*-cresol, in addition to being a substrate and thereby a competitive inhibitor with respect to tyramine, is a mechanism-based inhibitor of DBH (Goodhart et al., 1987). Inactivation results from covalent modification of the enzyme as evidenced by irreversible incorporation of radiolabeled *p*-cresol. Insertion of an aberrant benzylic radical intermediate into an active site residue was suggested to account for inactivation. Here we report the results of structural studies on DBH inactivated by *p*-cresol. Isolation and sequence analysis confirms that a single, major tryptic peptide is covalently modified by *p*-cresol. The structures of these adducts provide direct evidence for a benzylic radical intermediate in the inactivation event with *p*-cresol and therefore the first direct confirmation of a proposed hypothesis for the inactivation of DBH by a mechanism-based inhibitor.

Experimental Procedures

Materials. The following were obtained from Aldrich: L-ascorbic acid, *p*-cresol, disodium fumarate, *N*-ethylmorpholine (4-ethylmorpholine), iodoacetic acid, Polybrene, sodium ascorbate, and trichloroacetic acid. Prior to use, the *N*-ethylmorpholine was refluxed for 1 h with excess ninhydrin and then distilled from KOH. 3-Mercapto-1,2-propanediol (monothioglycerol) and tyramine hydrochloride were obtained from Sigma. Guanidine hydrochloride and trifluoroacetic acid were purchased from Pierce. Crystalline catalase was the product of Boehringer Mannheim. [3-^{3}H]-*p*-cresol was synthesized as reported by Goodhart et al. (1986). 2-Mercaptoethanol was obtained from Bio-Rad. Trypsin-TPCK was purchased from Cooper Biomedical, and the HPLC-grade solvents were supplied by J. T. Baker. DBH was prepared as previously described (Scott et al., 1988). All other chemicals were commercial products of the highest purity available.

Assay for DBH Activity. The activity of DBH was measured at 37 °C in standard 1-mL reaction mixtures containing 0.2 M sodium acetate, pH 5.0, 10 mM L-ascorbic acid, 10 mM disodium fumarate, 10 mM tyramine hydrochloride, 1 mg/mL catalase, and 10 μM cupric acetate. The reaction was initiated by the addition of an appropriate dilution of DBH stock solution and terminated by the addition of 0.2 mL of 3.0 M trichloroacetic acid. The octopamine produced was determined by a periodate cleavage method similar to that reported by Nagatsu and Udenfriend (1972).

Inactivation of DBH with p-Cresol. DBH was completely inactivated at 37 °C with *p*-cresol in a beaker containing 0.2 M sodium acetate, pH 5.5, 10 mM *p*-cresol, 10 mM sodium ascorbate, 10 μM $CuCl_2$, and 1 mg/mL catalase in a final volume of 10 mL. The concentration of DBH was 0.2 mg/mL (2.68 μM), and the inactivation was initiated by the addition of ascorbate. To prevent loss by evaporation during the 3-h incubation, the beaker was covered with perforated parafilm. During inactivation, the sample was agitated in a Model R-76 reciprocating shaker bath (New Brunswick Scientific) operating at 2 Hz to ensure atmospheric equilibration. The activity of the DBH was determined before and immediately following the incubation period by diluting 5 μL of the inactivation sample into the 1-mL standard assay mixture described above. In order to prepare a sufficient quantity of the peptides to complete all of the studies reported here, the inactivation was scaled up and repeated as necessary with nonradiolabeled *p*-cresol.

Isolation of the Inactivated DBH. Following inactivation, the sample was cooled on ice and then centrifuged at 10000*g* for 20 min to pellet the nonsolubilized catalase. The supernatant was removed and concentrated to approximately 1 mL in a Centricon-30 ultrafiltration apparatus (Amicon). The concentrated sample was then centrifuged for 2 min in an Eppendorf Microfuge (Brinkmann Instruments) to pellet any remaining nonsolubilized catalase. Separation of the DBH from the soluble catalase and low molecular weight components was achieved by size-exclusion chromatography on an analytical Bio-Sil TSK-250 HPLC column (7.5 × 300 mm, Bio-Rad Laboratories) equilibrated with 5 mM potassium phosphate, pH 6.5–0.2 M KCl at a flow rate of 1 mL/min. The pooled fractions of purified DBH collected following multiple 250-μL injections were dialyzed against 2 × 2 L of 5 mM NEMAc, pH 8.5, and dried at ambient temperature on a Savant Instruments Speed Vac concentrator. Complete separation of the DBH and catalase by this method was confirmed by SDS–PAGE.

S-Carboxymethylation and Tryptic Digestion. Reduction and carboxymethylation of the purified DBH was performed by the method of Crestfield et al. (1963) using 6 M guanidine hydrochloride instead of 8 M urea (Marcus et al., 1981). The iodoacetic acid was neutralized by adding 0.5 mol equiv of Na_2CO_3 rather than 1 mol equiv of NaOH. The reduced, carboxymethylated sample was dialyzed in the dark at 4 °C against 2 L of 0.1 M ammonium acetate and then 5 × 2 L of deionized water with a minimum of 2 h between changes. The sample was then dried on a Speed Vac concentrator. Digestion of the S-carboxymethylated protein was carried out for 16 h at 22 °C in 100 mM NEMAc, pH 8.5, with trypsin-TPCK at a 50:1 ratio of DBH to trypsin. Following digestion, the sample was acidified to pH 6 by the addition of 50% TFA and then centrifuged for 2 min in an Eppendorf Microfuge to pellet any insoluble matter.

Reversed-Phase HPLC of Tryptic Peptides. The tryptic peptides were fractionated at 22 °C by reversed-phase HPLC using a Vydac Protein C_4 column (4.6 × 250 mm, 5 μm, Separations Group) fitted with a 3-cm Aquapore RP-300 guard cartridge (7 μm, Brownlee Labs). The column was equilibrated with 0.1% TFA at a flow rate of 0.5 mL/min. Elution of the peptides was achieved with an H_2O/CH_3CN gradient (0–63% CH_3CN over a period of 160 min) containing 0.1% TFA as indicated in Figure 1. The radiolabeled peptides were further fractionated at 40 °C on a Vydac Phenyl column (4.6 × 150 mm, 5 μm, Separations Group) equipped with a 3-cm Aquapore PH-300 guard cartridge (7 μm, Brownlee Labs) equilibrated at 1 mL/min with 7% CH_3CN in H_2O containing 0.1% TFA. The peptides were eluted with a linear CH_3CN/H_2O gradient from 7 to 70% CH_3CN at a rate of 0.5%/min.

Conventional Sequencing of Tryptic Peptides. Automated Edman degradation of the major peptides was performed on a Beckman 890 M sequencer using the PPMMS program. Prior to sample application to the sequencer, 1 mg of Polybrene was applied to the spinning cup, and one cycle without conversion was performed. The Pth-amino acids were analyzed on a Beckman Ultrasphere ODS column (4.6 × 250 mm, 5 μm)

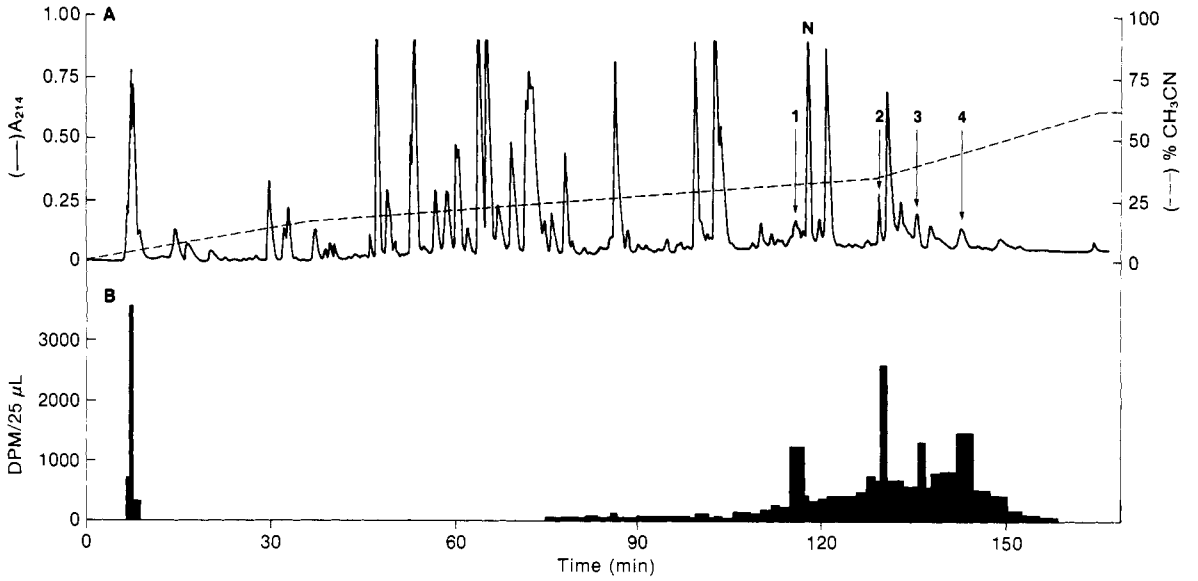


FIGURE 1: Reversed-phase HPLC profile of a tryptic digest of *p*-cresol-inactivated DBH. Approximately 10 nmol of the acidified tryptic supernatant was injected onto a Vydac Protein C_4 column (4.6 × 250 mm) equilibrated at a flow rate of 0.5 mL/min with 0.1% aqueous TFA. A gradient of 0–63% CH_3CN was used to elute the peptides which were collected manually. Panel A shows the resultant chromatogram at 214 nm (—) and the gradient program used (– –). Panel B is a histogram showing the distribution of the radioactivity across the chromatogram obtained by counting 25 μL of each fraction. Since the widths of the bars were adjusted to correspond to the volumes of the fractions, area rather than bar height is a more accurate representation of the amount of radiolabel in each fraction. The numbered arrows (panel A) indicate fractions containing radiolabeled peptides. The peak labeled N is the native (unmodified) peptide corresponding to peptides 2 and 3.

at 50 °C with a trifluoroacetic acid–acetate gradient essentially as described by Hawke et al. (1982). In order to establish the site of covalent modification, approximately 20% (10 μL) of the Pth-amino acid from each cycle following Edman degradation was counted with 5 mL of Aquasol-2 (Beckman Instruments). Automated Edman degradation of the minor peptides (10–75 pmol) was performed on an Applied Biosystems 477A protein sequencer equipped with the 120A online Pth analyzer, and the sample disks were loaded with Polybrene prior to sample application.

Purification of Pth-Amino Acids. Prior to mass spectral analysis, certain Pth-amino acids were recovered following Edman degradation of the peptides and purified by reversed-phase HPLC at 40 °C on a Beckman Ultrasphere ODS column (4.6 × 250 mm, 5 μm) fitted with a 3-cm Spheri-5 RP-18 guard cartridge (7 μm, Brownlee Labs). A linear gradient from 27 to 72% CH_3CN/H_2O containing 0.1% TFA was used to elute the Pth-amino acids. The flow rate was 1 mL/min, and the rate of change of the gradient was 0.9%/min.

Mass Spectral Analysis of Tryptic Peptides and Pth-Amino Acids. Conventional FAB mass spectra (magnetic scan on a double-focusing instrument) were obtained by use of MS-1 of a VG ZAB SE-4F tandem mass spectrometer equipped with a standard FAB ion source and an Ion Tech fast atom gun (Carr et al., 1988). A VG 11-250J data system was used to acquire and process all data. Approximately 1 nmol of the blocked tryptic peptide was dissolved in 20 μL of 20% CH_3CN/H_2O/0.1% TFA, and about 2 μL of the resulting solution was dispersed on the stainless steel target in a matrix of 3-mercapto-1,2-propanediol. The accelerating voltage of the mass spectrometer was maintained at 10 kV while 8-keV xenon atoms at a discharge current of 1 mA were used to bombard the sample. Spectra were acquired at a resolution of 1000, scan rate of 150 s/dec over the mass range of 4000–1000. Raw peak profiles were acquired, and three to five scans were summed prior to centroiding and mass assignment.

The tandem MS experiments were performed with a VG ZAB SE-4F, a four-sector instrument of B_1E_1-C-E_2B_2 configuration where B and E signify magnetic and electric sectors, respectively, and C represents the collision region. Sample preparation and ionization were as described above. Parent ions in the chymotryptic digest (3 h, 37 °C, in 50 mM NH_4HCO_3, enzyme:substrate = 1:50 w/w) of peptide 2 were sequentially selected with MS-1. The pressure of He in the collision region between the two mass spectrometers was adjusted so as to attenuate the parent beam selected with B_1E_1 by 50%. Daughter ion spectra were obtained by an E_2/B_2-linked scan. The mass scale of the second mass spectrometer was calibrated in the FAB mode by use of an intermediate ion source located between MS-1 and MS-2. All data were acquired with the VG 11-250J data system operating in the raw data accumulation mode with a scan rate of 30 s/dec. Under these conditions, between three and six scans could be accumulated per loading of sample (approximately 350 pmol).

Desorptive chemical ionization (DCI) MS was performed on a Finnigan-MAT 4610 quadrupole mass spectrometer equipped with an INCOS data system. Approximately 0.25 nmol of each amino acid derivative was loaded via microsyringe onto the DCI wire loop. A heating rate of 20 mA/s was employed. Ammonia and deuteriated ammonia (Cambridge Isotope, >99.5% ^2H) were used as the reagent gases. Model studies to define the number of exchangeable protons in the Pth-amino acids during N^2H_3-DCI MS were carried out with between 1 and 5 μL of standard solutions of Pth-Tyr, -Lys, -Gly, and -Leu at concentrations of 0.1 nmol/μL in CH_3CN/H_2O, 1:1 (v/v).

RESULTS

Reversed-Phase HPLC of the Tryptic Digest. As shown in Figure 1A, reversed-phase HPLC separation of the tryptic peptides obtained from S-carboxymethylated, *p*-cresol-inactivated DBH yields a pattern consisting of approximately a dozen major and numerous minor peaks. The distribution of radioactivity across the chromatogram (Figure 1B) reveals, in addition to a peak of counts eluting in the break-through fraction, four peaks of radioactivity that appear to correspond to distinct peaks of material absorbing at 214 nm (Figure 1A).

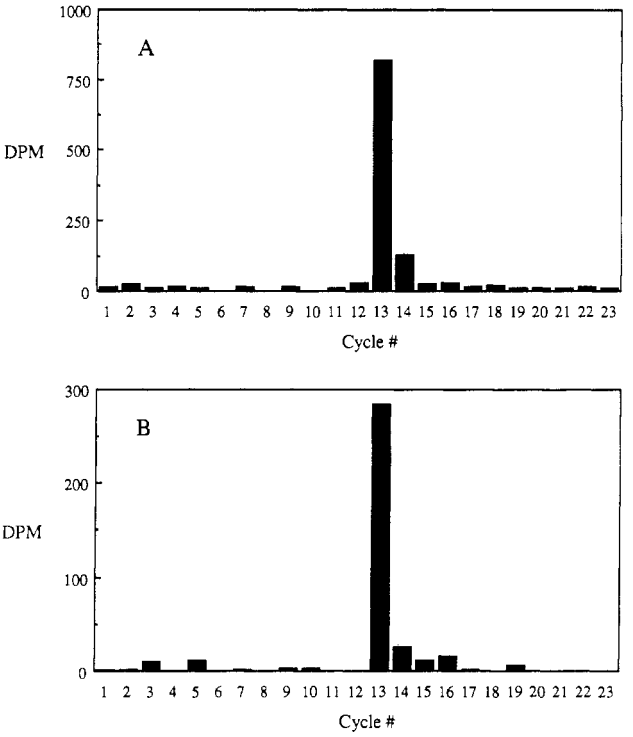


FIGURE 2: Histograms showing the results from counting 20% of the Pth-amino acids from each cycle of the Edman degradation of peptides 2 (panel A) and 3 (panel B). The modified residue is indicated by the tall bar in each panel.

Table I: Recovery of Radioactivity following Tryptic Digestion and Reversed-Phase HPLC of *p*-Cresol-Inactivated DBH

sample	estimated dpm_T	% recovery
tryptic digest	1 680 000	100
acidified tryptic supernatant	870 000	52
C_4 column eluate	703 000	81[a]
C_4 break-through fraction	53 000	7.5[b]
peak 1	57 400	8.2[b]
peak 2	53 500	7.6[b]
peak 3	32 700	4.7[b]
peak 4	79 300	11[b]

[a] Represents percent recovery of total radioactivity injected onto the C_4 column. [b] Represents percent of total radioactivity recovered in C_4 column eluate.

These radiolabeled peaks, designated 1–4, are situated atop a broad hump of radiolabel that makes up the majority of the recovered counts and does not appear to be specifically associated with any distinct peptide peaks. A comparison of the tryptic map in Figure 1A to that produced from native DBH (not shown) reveals that peaks 2 and 3 are present only in the *p*-cresol-inactivated sample.

The four peaks containing radiolabeled peptides constitute only a relatively small portion of the radioactivity originally present in the tryptic digest. An analysis of the recovery of radiolabel at each step following tryptic digestion is given in Table I. When the tryptic digest is acidified to pH 6 with 50% TFA and centrifuged, only about half of the radioactivity is found in the supernatant. The remainder is located in an "acid-insoluble" pellet that consists primarily of a large 10–20-kDa DBH fragment that is refractory to digestion by trypsin (data not shown). Of the total radiolabel recovered following C_4 column chromatography, approximately one-third is contained in peaks 1–4, 7.5% is found in the break-through fraction, and the remaining 60% is eluted nonspecifically as a very broad peak spanning approximately 60 min. Because they clearly represent novel peaks resulting from inactivation and they appear to be single, discrete peptides, peaks 2 and 3 were selected for detailed study.

Conventional Sequencing of Peptides 2 and 3. Following further purification by reversed-phase refractionation on a Vydac Phenyl column, peptides 2 and 3 were submitted to conventional sequencing via Edman degradation that resulted in the following partial sequences:

Peptide 2

5 10
Ala-Pro-Asp-Val-Leu-Ile-Pro-Gly-Gln-Gln-Thr-Thr-

15 20
xxx-Trp-Cys-Tyr-Val-Thr-Glu-Leu-Pro-Asp-Gly- . . .

Peptide 3

5 10
Ala-Pro-Asp-Val-Leu-Ile-Pro-Gly-Gln-Gln-Thr-Thr-

15 20
xxx-Trp-Cys-Tyr-Val-Thr-Glu-Leu-Pro-Asp-Gly- . . .

The sequence could not be determined beyond residue 23 due to the rapid disappearance of the signal when the Beckman 890M sequencer was used. However, it appeared likely that both peptides were identical with the exception of the modification at residue 13. To confirm this, a portion of the Pth-amino acid from each cycle was tested for its radioactive content. The result of this experiment (Figure 2) reveals that in both cases most of the radiolabel occurs in cycle 13.

Mass Spectral Studies on Peptides 2 and 3. FAB mass spectra utilizing approximately 150 pmol of each peptide yielded chemical average molecular weights of 3131.2, further supporting the identity of the two peptides. In order to verify the Edman-derived sequence and to provide independent support for the modification of residue 13, approximately 450 pmol of each peptide was digested briefly with chymotrypsin, and the resulting digests were analyzed directly by FABMS. The choice of enzyme was dictated by the Edman sequence data that indicated two prime chymotryptic sites at residues 14 (Trp) and 16 (Tyr). Both peptides yielded identical spectra exhibiting four peptide signals with MH_{av} of 1131.3, 1455.6, 1695.8, and 2019.8. These data suggest that the peptides have the same sequence and that their difference is due to isomeric modification of residue 13.

None of these peptides can be assigned simply on the basis of the Edman data. However, several points can be established by simple arithmetic manipulation of the observed masses. First, the sum of the 1455 and 1695 MH's minus the mass corresponding to H_3O[2] equals the observed MH of the intact peptides. Therefore, these two signals represent the two halves of the modified peptides. Similarly, the 1131 and 2019 signals also sum (after subtracting H_3O) to the observed MH for the intact tryptic peptides. Second, the pairs of signals at m/z 1131/1455 and 1695/2019 each differ by 324 Da, which is the in-chain mass of CMCys-Tyr, the two residues that are bounded by the prime chymotryptic cleavage sites. Third, according to the Edman data, the higher mass peptide signals at 1695 and 2019 must derive from the amino termini of the peptides while 1131 and 1455 come from the carboxyl termini. Finally, subtraction of the sum of the in-chain masses of the amino acids known to be present in peptide 1–14 from the determined mass (1695) yields a mass of 269 Da for the modified amino acid. Subtraction of 106, the residue mass of *p*-cresol, from 269 yields 163, the in-chain mass of tyrosine.

[2] Not only must the mass of H_2O be subtracted to account for the peptide bond but also one additional mass unit since there is one fewer MH when the intact peptide is considered. Therefore, 1455 + 1695 − 19 = 3131.

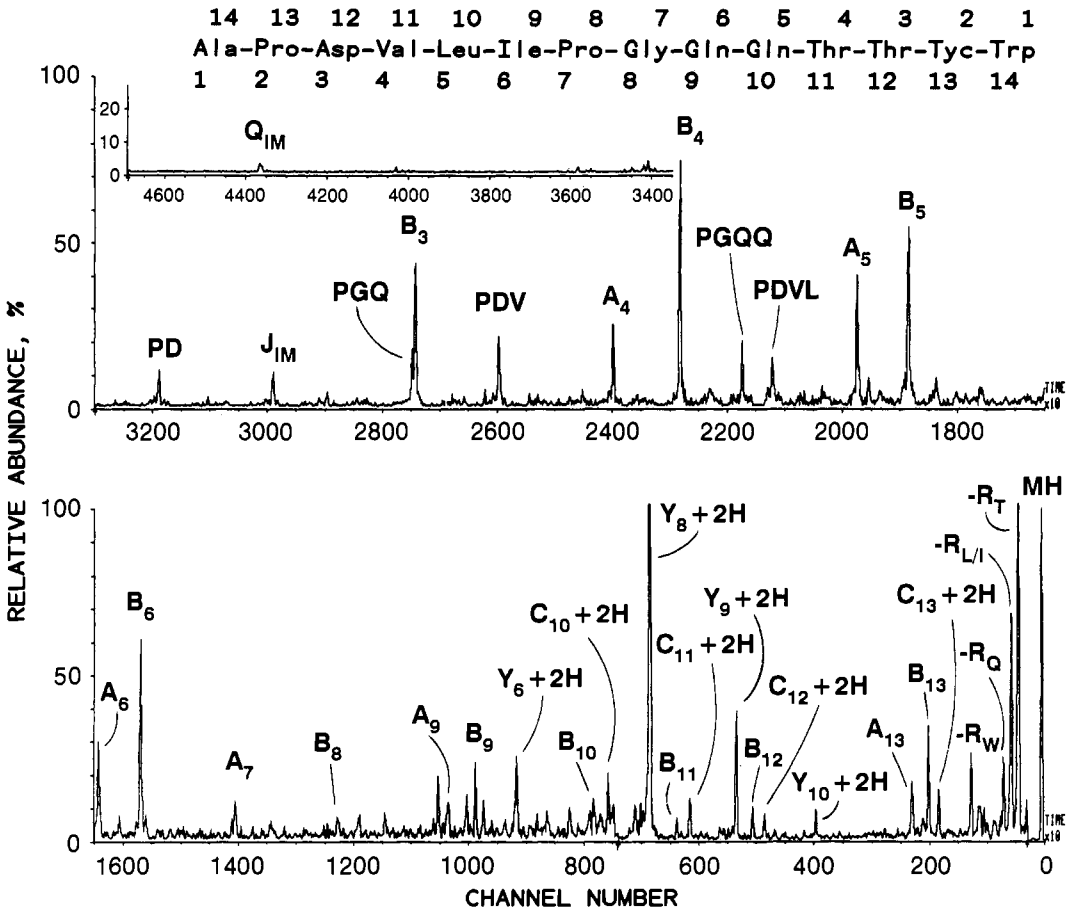


FIGURE 3: Tandem mass spectrum of the chymotryptic peptide of $(M + H)^+_{av}$ = 1695.9. FABMS in MS-1 of the VG ZAB SE-4F tandem mass spectrometer was used to identify this parent ion in the mixture of peptides resulting from chymotryptic digestion of peptide 2 (see Figure 1) and to select it for collision with He at 10 keV (pressure sufficient to reduce parent to 25% of original intensity) and mass analysis of the resulting product ion in MS-2. Numbering above the printed three-letter code sequence refers to carboxyl-terminal ions (V–Z), while that below the printed sequence refers to amino-terminal ions (A–C). Fragment ion nomenclature is according to Roepstorff and Fohlman (1984) and Johnson et al. (1987). A_n, B_n, and C_n ions are due to peptide backbone fragment ions and correspond to cleavage of the $NHCHR_n$–CO, CHR_nCO–NH, and NH–CHR_{n+1} bonds, respectively, with charge retained on the amino-terminal fragments. X_n, Y_n, and Z_n ions (where n is in ascending order from the carboxyl terminus) also are due to peptide backbone fragment ions and correspond to cleavage of the CHR_{n+1}–CO, CO–$NHCHR_n$, and NH–CHR_n bonds, respectively, with charge retained (with or without H transfers as indicated) on the carboxyl-terminal fragments. Secondary fragment ions, labeled W_n and V_n, also are observed and are due to side-chain losses. W_n fragment ions originate from the Z_n + H ions by β–γ cleavage of the side chain of the nascent amino-terminal residue. V_n fragment ions come from the Y_n + 2H ions by loss of RH, where R is the entire side chain of the nascent amino-terminal residue. J = single-letter representation of tyrosine with *p*-cresol covalently attached; Tyc is the three-letter code used for this residue. PGQ, PDV, PGQQ, and PDVL are internal fragments (Johnson et al., 1987).

Thus, the unknown residue occurring at cycle 13 in the Edman sequencing data is likely a *p*-cresol-modified tyrosine.

In order to unambiguously establish the nature of residue 13 as *p*-cresol-modified tyrosine and to determine the complete sequence of peptides 2 and 3, each of the peptides in a chymotryptic digest of about 1 nmol of the tryptic peptide was analyzed by tandem MS directly from the mixture without isolation or purification of the individual peptides. In the tandem mass spectra of peptides, sequence-defining "primary" fragments are formed by cleavage of the amide backbone, with or without H rearrangement, resulting in ion series derived from the carboxyl terminus (X_n, Y_n, and Z_n, Figures 3 and 4) and complementary series of fragments (A_n and B_n) from the amino terminus (Johnson et al., 1987). Secondary fragment ions labeled W_n and V_n (Figure 4) are also commonly observed (Stultz & Watson, 1987; Johnson et al., 1987). Other fragment ions that are commonly observed in the tandem mass spectra of peptides include loss of amino acid side chains from the $(M + H)^+$ (e.g., $-R_T$, Figure 3) and immonium ions $(H_2N{=}CHR)^+$ (e.g., Q_{im}, m/z 101; Figure 3) where R is the amino acid side chain. The tandem mass spectrum of the 1695 peptide (Figure 3) is dominated by amino-terminal sequence ions that define the sequence (Ala,Pro,Asp)-Val-Leu-Ile-Pro-Gly-Gln-Gln-Thr-Thr-Tyc-Trp, where Tyc is apparently a modified amino acid residue with an in-chain mass equivalent to the sum of the incremental masses of a Tyr plus cresol (each having lost a H to form a covalent bond), i.e., 163 + 108 − 2 = 269. The mass of Tyc is defined by the mass difference from sequence ions C_{13} to C_{12} and B_{13} to B_{12} (Figure 3). The tandem mass spectrum of this peptide could not be used to independently establish the sequence of the first three residues because amino-terminal sequence ions for the first two residues (i.e., A_n and B_n, where n = 1 and 2) were not observed. Of course, this is also the amino terminus of the peptide where the Edman data are most reliable. In a similar fashion we have sequenced the two carboxyl-terminal peptides in the chymotryptic digest with MH's of 1131 (not shown) and 1455 (Figure 4) which enabled us to elucidate the remaining carboxyl-terminal sequence not accessible by the Edman method. The sequence of the 1455 peptide, therefore, was found to be Cys-Tyr-Val-Thr-Glu-Leu-Pro-Asp-Gly-Phe-Pro-Arg. A summary of the sequence information provided in the various chemical and mass spectral experiments is shown in Figure 5.

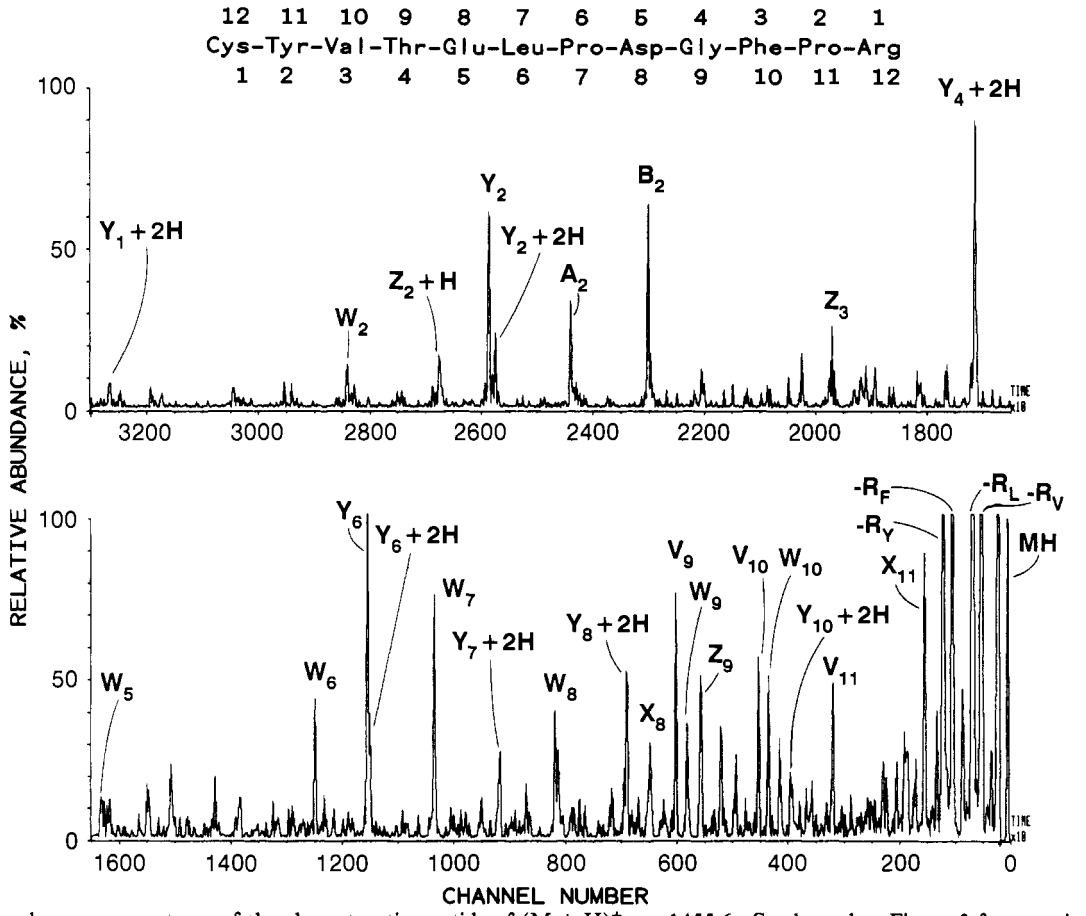


FIGURE 4: Tandem mass spectrum of the chymotryptic peptide of $(M + H)^+_{av}$ = 1455.6. See legend to Figure 3 for experimental details.

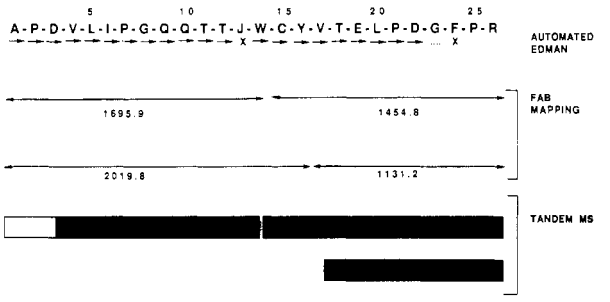
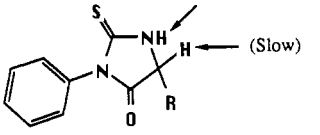


FIGURE 5: Summary of the sequence information derived for peptides 2 and 3 (Figure 1). Single-headed arrows represent cycles of automated Edman degradation; X signifies an unidentified residue. Double-headed arrows indicate the sequence origins of the signals observed in the chymotryptic digests of peptides 2 and 3 by FABMS. Rectangles refer to peptides analyzed by tandem MS; blackened regions signify sequences established by tandem MS. The sequence of the first three residues of the peptide could not be unambiguously established by tandem MS (open box). J = single-letter representation of tyrosine with *p*-cresol covalently attached.

Gas-Phase Deuterium Exchange Studies. The linkage of *p*-cresol to the tyrosine at residue 13 was examined by N^2H_3-DCI MS of the Pth-amino acids from the Edman degradation of peptides 2 and 3. The "core" of a Pth-amino acid exhibits two exchangeable protons in the N^2H_3-DCI mass spectrum (Scheme II): a rapidly exchanging secondary amine proton and a slowly exchanging methine proton on the α-carbon. Exchangeable protons in the side chain of the amino acid residue are added to the number of core exchangeables. The mass spectra of Pth-Tyr analyzed according to this procedure (not shown) indicate the presence of two and three exchangeable protons—two from the Pth core and one phenolic proton in the R group. The corresponding DCI mass spectra

Scheme II[a]



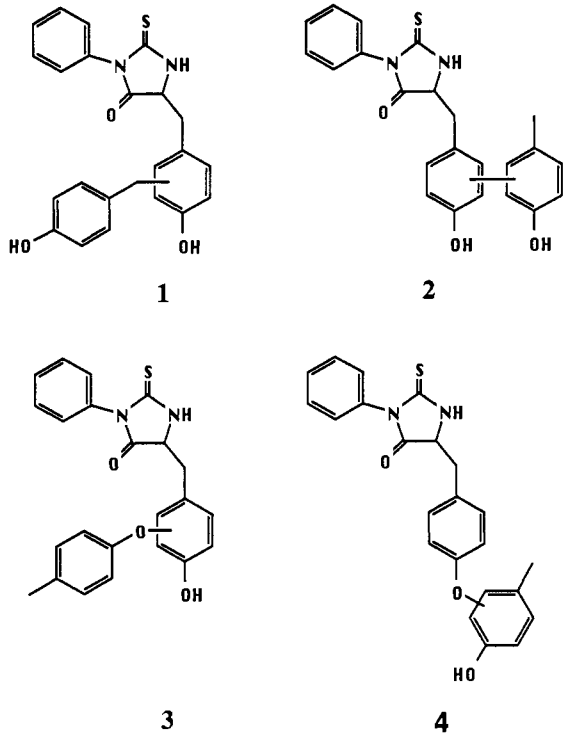
[a] Arrows indicate exchangeable protons.

of cycle 13 from the Edman degradation analyses of peptides 2 and 3 reveal the presence of 3/4 and 2/3 exchangeable protons, respectively (Figure 6). These results suggest that *p*-cresol is linked through its methyl group or the phenyl ring in peptide 2 forming [(4-hydroxyphenyl)methyl]-Tyr's (i.e., the R group has *two* free hydroxyl groups; see structures 1 and 2) while the cresol is linked through an oxygen atom in peptide 3 to give [(4-methylphenyl)oxy]-Tyr's (i.e., the R group has only *one* free hydroxyl group; see structures 3 and 4).

Conventional Sequencing of the Minor and Native Tryptic Peptides. As part of a total DBH sequence determination, all the major tryptic peptides shown in Figure 1 were sequenced. This led to the identification of peak N as the native, unmodified peptide corresponding to peptides 2 and 3. It was possible to determine the entire sequence for this peptide with the Applied Biosystems protein sequencer. Trace quantities (10–50 pmol) for four other minor radiolabeled peptides isolated from peaks 1 and 4 were sequenced. All but one have sequences identical with those of peptide 2 and 3 for residues 1–12, and in two of the peptides, "holes" appear at residue 13 suggesting additional modes of alkylation. The very limited quantities of these peptides have precluded extensive analysis.

Discussion

In this paper we have described the synergistic use of MS, tandem MS, and conventional Edman sequencing strategies



to identify the attachment site of the mechanism-based inhibitor *p*-cresol in the sequence of DBH. We have characterized two modified, 26 amino acid tryptic peptides from *p*-cresol-inactivated DBH and have been able to structurally define the covalent adducts resulting from alkylation of a tyrosine residue at position 13. The instrumental, chemical, and biochemical approaches were used so as to take advantage of their unique strengths. Specifically, peptide mapping by FABMS was used to rapidly check the overall correctness of the deduced sequence and to define the region of the peptide where the modification was present. The chymotryptic fragments observed by FABMS were analyzed without extensive cleanup by tandem MS to provide the sequence of the three amino acids at the carboxyl terminus of the peptide that were not initially accessible by Edman degradation. All of the mass spectral studies reported here were performed on only 2–3 nmol of peptide, which highlights the sensitivity of the FABMS and tandem MS methodology. Finally, O- versus C-linkage of *p*-cresol to tyrosine was established by determining the number of protons on the side chain of the modified amino acid that could be exchanged in the gas phase inside the ion source of a mass spectrometer. This experiment is a simple extension of conventional chemical ionization MS and was performed with a few hundred picomoles of material. Establishing the nature of the linkage by other spectroscopic techniques with such small amounts of material would have been difficult if not impossible.

Independently of our studies, Fitzpatrick and Villafranca (1987)[3] published the sequence of a 26 amino acid "active site" tryptic peptide which differs from our sequence by two residues. These authors report Gln-Cys in positions 15 and 16 rather that our reported sequence of Cys-Tyr. On the basis of our conventional Edman sequencing of *all* the major tryptic peptides in Figure 1, we conclude that a second 26 amino acid tryptic peptide with Gln-Cys at residues 15 and 16 is highly

[3] This citation refers to a note added in proof in which the authors, citing unpublished observations, report the sequence of the 26 amino acid peptide. No information is given regarding the method used to obtain the sequence or how they concluded that it is an active site peptide.

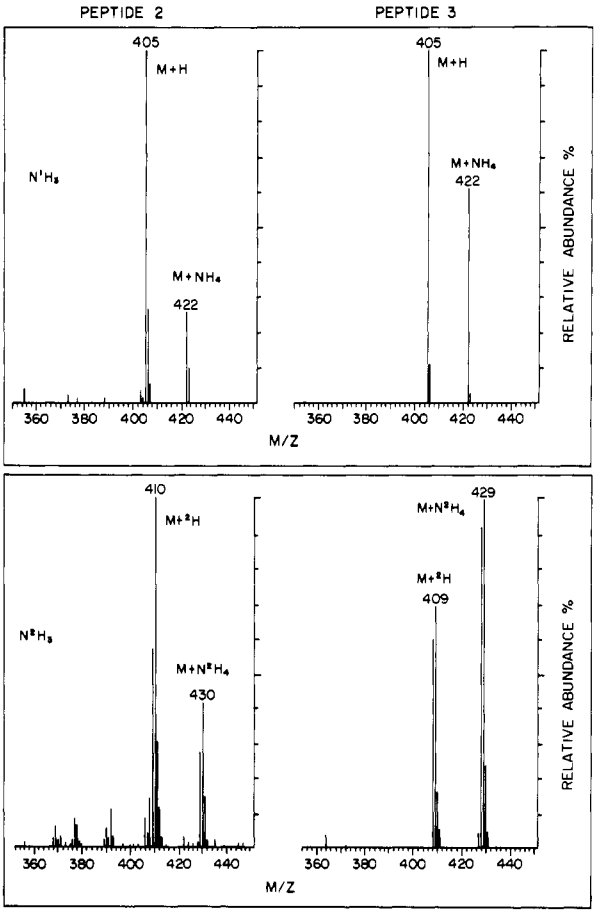


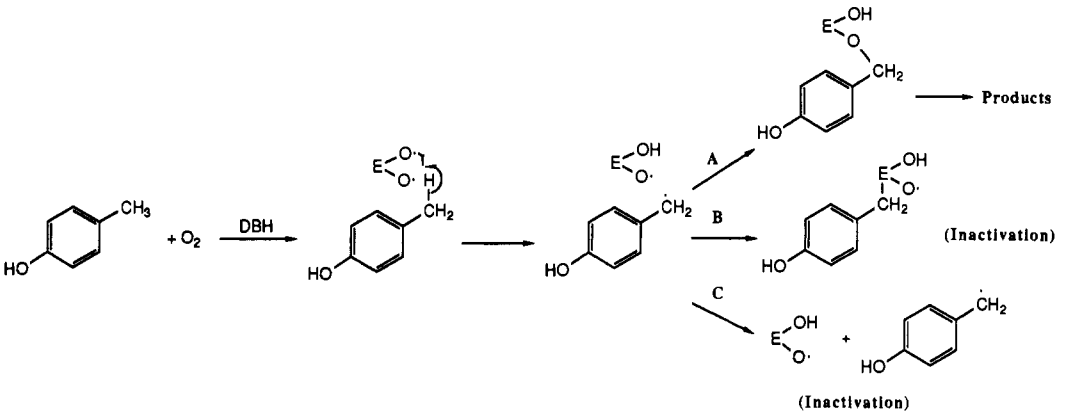
FIGURE 6: Desorptive chemical ionization mass spectra of the HPLC-purified Pth-amino acids from cycle 13 of peptides 2 and 3 using (top panel) N^1H_3 and (bottom panel) N^2H_3 as the reagent gasses. Spectra for peptide 2 are on the left in each panel while those for peptide 3 are on the right. The complexity in the pseudomolecular ion clusters exhibited when N^2H_3 is used as the reagent gas is due to relatively slow exchange of the methine proton of the α-carbon of the amino acid (see text).

improbable. We believe that the combination of Edman and mass spectral data given here provides unequivocal evidence for the sequence we have described.

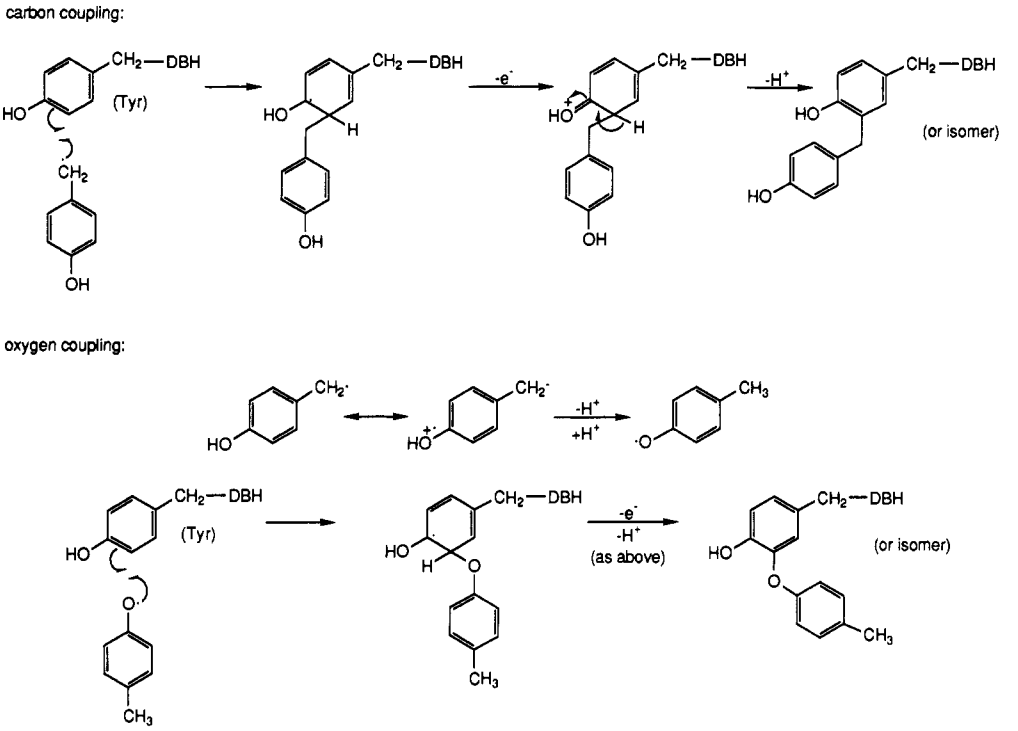
Scheme III depicts our view of the chemical events leading from the activation of *p*-cresol through the reactive intermediate (benzyl radical) and on to either products or inactivation. The pathway yielding the product 4-hydroxybenzyl alcohol (A) is a general mechanism similar to that suggested by other investigators (Miller & Klinman, 1985) for physiological substrates. The pathway leading from the benzyl radical to inactive enzyme (B) is similar to the one we have proposed earlier (Goodhart et al., 1987). Our identification of *p*-cresol-modified Pth-tyrosines as the products formed during inactivation by *p*-cresol is consistent with a mechanism of inactivation where insertion of the initially formed (or a rearranged) radical leads to the covalently modified tyrosines. Scheme IV shows two of the possible mechanisms whereby a *p*-cresol-derived benzyl radical could lead to either a carbon- or oxygen-linked modified tyrosine.

It is tempting to speculate that the tyrosine that is modified functions to assist the binding of the substrate in the active site via a π–π interaction with the phenyl group of the phenethylamine. Support for this concept might be provided by demonstrating that this tyrosine is conserved among different species. The recently reported sequence of human DBH (Lamouroux et al., 1987) reveals a region corresponding to peptides 2 and 3 that encompasses residues 204–229. Although

Scheme III



Scheme IV



the overall homology of that sequence to peptides 2 and 3 is only 65%, the hexapeptide including residues 11–16 and containing the putative active site tyrosine is identical with the human sequence, demonstrating that this is a highly conserved (and therefore critical?) region. This homology also supports our previous assignment of Cys-Tyr to residues 15 and 16.

As previously reported (Goodhart et al., 1987), the stoichiometry of incorporation by *p*-cresol is considerably less than one. This led us to propose an alternate mechanism for the inactivation involving autoxidation by a copper–oxygen radical (Scheme III, pathway C) left behind by the dissociation of the benzyl radical from the active site. In support of this hypothesis, we have identified the native (unmodified) tryptic peptide corresponding to peptides 2 and 3 in digests of *p*-cresol-inactivated DBH (Figure 1, peak N), where virtually all of the activity has been lost. A qualitative comparison of the relative areas of the unmodified (N) and the modified (1 through 4) peptides supports our previous finding that the stoichiometry of incorporation is substantially less than one.

The inactivation of DBH by *p*-cresol is somewhat surprising given the fact that this compound lacks a latent electrophilic functional group, which is normally considered to be prerequisite in the design and action of mechanism-based inhibitors. Although this finding is unusual, it is by no means unique. We recently reported a number of other, related compounds which inactivate DBH with varying degrees of efficacy (Goodhart et al., 1987). These include *m*-cresol, 4-methylcatechol, 4-ethylphenol, and 3-hydroxybenzyl alcohol. 4-Ethylphenol is interesting in that it is a close analogue of tyramine lacking only a side-chain amino group. It thus appears that, for compounds of this type, the amino group plays a significant role in distinguishing a substrate from an inactivator, and any proposed mechanism for the inactivation by these compounds should necessarily account for this observation. The close structural relationship these have to conventional, physiological substrates (dopamine and tyramine) suggests that inactivation is the result of an ill-fated, reactive intermediate (benzyl radical) produced during the normal turnover of substrate to product. If this is true, it follows that the potential for mechanism-based inhibition should exist in the phenethylamine substrates as well and that the distinction between whether a compound will behave as either a substrate or an inactivator will depend on the relative ratio of the rate constants leading to the breakdown of the reactive intermediate, resulting in either the formation of products or inactivation of the enzyme

(Scheme III). It would thus appear that DBH and perhaps other monooxygenases which employ radical chemistry during normal catalysis operate in a reaction manifold that is kinetically close to potential inactivation events. Seemingly minor structural modifications such as deletion of the basic nitrogen are sufficient to convert a DBH substrate to a potent mechanism-based inhibitor. It is interesting to note that a similar tendency has been observed for alternate substrates of the copper-containing oxidase tyrosinase (K. Lerch, personal communication). We have observed that when we run prolonged assays with tyramine as the substrate there is a gradual decrease in the rate of product formation over time. Intuitively, we have always attributed this decline to either product inhibition or to autoxidation of the enzyme by peroxide, but we now question whether inactivation by tyramine itself might be involved. To the best of our knowledge, this possibility has never been considered or tested.

The role of the positively charged amino group in preventing inactivation by phenethylamine substrates may lie in its ability to destabilize the benzyl radical intermediate by inductive electron-withdrawing effects, thereby promoting radical recombination with oxygen at the β-carbon. The more stable radical formed by hydrogen atom abstraction from *p*-cresol is more likely to reorient in the active site leading to alkylation (pathway B) or to dissociate from the active site prior to condensation with oxygen causing inactivation by pathway C (Scheme III).

The amino group does not appear to provide any additional binding affinity for the substrate as evidenced by the fact that the K_m(app) for tyramine is not significantly different from the K_I(app)'s of 4-ethylphenol and *p*-cresol (Goodhart et al., 1987). We have recently discovered that β-ethynyl- and β-vinyltyramine exhibit K_{is}'s 10–100-fold lower than the K_m for tyramine (Kruse et al., 1988). This suggests that there is a hydrophobic pocket adjacent to the β-carbon capable of interacting with and increasing the affinity of these phenethylamine analogues.

In conclusion, we have provided evidence that *p*-cresol, a compound that lacks a latent electrophile, inactivates DBH by a mechanism involving alkylation of the enzyme by a reactive intermediate produced during the normal turnover of substrate to product. This inactivation appears to be closely linked to the lack of a positively charged amino group on the substrate. In view of the fact that most of the reported mechanism-based inhibitors of DBH also lack this functionality (Fitzpatrick & Villafranca, 1987), it is clear that additional kinetic or structural evidence is needed to confirm the involvement of the intrinsic, latent electrophilic functional groups in the inactivation by these compounds.

ACKNOWLEDGMENTS

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Registry No. DBH, 9013-38-1; L-Tyr, 60-18-4; *p*-cresol, 106-44-5.

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