Complete Assignment of Disulfide Bonds in Bovine Dopamine β-Hydroxylase†

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ABSTRACT: Peptide mapping, chemical sequencing, microbore HPLC/electrospray ionization mass spectrometry (LC/ESI/MS),[1] and matrix-assisted laser desorption mass spectrometry (MALDI/MS) were used to identify the sites of intra- and intermolecular disulfide linkages in bovine dopamine β-hydroxylase. The enzyme contains 14 cysteines and seven disulfides per monomer. Edman sequencing of tryptic and peptic peptides determined linkages at positions Cys140–Cys582, Cys218–Cys269, Cys255–Cys281, Cys452–Cys474, Cys514–Cys514, and Cys516–Cys516, where cysteines at positions 514 and 516 on one monomer disulfide pair with their homologs on a second monomer. These linkages were confirmed by LC/ESI/MS and MALDI/MS. Further analysis by LC/ESI/MS and MALDI/MS identified linkages at positions Cys376–Cys489 and Cys380–Cys551. Cysteines 140 and 582 form a disulfide linkage that folds the C-terminus back in proximity to the N-terminus. The remaining intramolecular disulfides occur along two separate internal regions of the protein. The density of histidine residues in these two regions suggests binding sites for two Cu^{2+} atoms per monomer. In addition, previously identified amino acids that react with mechanism-based inactivators occur in these two regions. We propose that these five internal disulfide bonds define two Cu^{2+} binding domains that make up the active site of a dopamine β-hydroxylase monomer. Considering previous data on the location of glycosylation sites, mechanism-based inactivation sites, and the disulfide linkages presented here, the data suggest an overall topology where the N- and C-termini are in close proximity and are solvent exposed and where the Cu^{2+} binding sites are buried in two interior domains stabilized by five disulfide bonds.

Dopamine β-hydroxylase catalyzes the physiologically important conversion of dopamine to norepinephrine. It is the third step in the catecholamine biosynthetic pathway and provides norepinephrine for the sympathetic nervous system. It is found primarily in the chromaffin granules of the adrenal gland. Extensive enzymatic and physical studies have described both the kinetic (Fitzpatrick et al., 1986; Ahn & Klinman, 1983) and chemical (Fitzpatrick & Villafranca, 1987) mechanisms of the enzyme, as well as the metal stoichiometry (Ash et al., 1984; Klinman et al., 1984) and metal geometry (Blumberg et al., 1989) at the active site. Carbohydrate analysis has shown that the enzyme contains a 2:1 ratio of biantennary complex oligosaccharides to high mannose oligosaccharides (Margolis et al., 1984). Biophysical studies have shown that two disulfide-linked dimers reversibly associate to form a tetramer (Saxena et al., 1985). Both membrane-bound and soluble forms of the enzyme have been purified (Aunis et al., 1977; Colombo et al., 1987). The properties of the enzyme have been reviewed (Villafranca et al., 1985; Stewart & Klinman, 1988).

The polypeptide sequence of the bovine enzyme has been determined by two groups (Robertson et al., 1990, 1991; Wang et al., 1990), and the genes for the human (Lamouroux et al., 1987; Kobayashi et al., 1989), bovine (Taljanidisz et al., 1989; Wang et al., 1990; Lewis et al., 1990), rat (McMahon et al., 1990), and mouse (Nakano et al., 1992) enzyme have been cloned. The cloned bovine enzyme has been expressed in PC12 cells (Lewis et al., 1992), COS cells (Ishii et al., 1991), and *drosophila* Schneider 2 cells (Gibson et al., 1993), but has not yet been overexpressed in large quantities. The successful expression of dopamine β-hydroxylase holds promise for site-specific mutagenesis and the prospect of even more detailed mechanistic studies.

Sequences derived from the cDNA have predicted an N-terminal signal peptide that is missing in the mature enzyme (Lewis et al., 1990; Taljanidisz et al., 1989), and Edman sequencing has demonstrated microheterogeneity at the N-terminus of mature soluble dopamine β-hydroxylase (Robertson et al., 1990; Wang et al., 1990; Taylor et al., 1989). Both of these results are consistent with evidence for a precursor–product relationship between the membrane-bound and soluble forms of dopamine β-hydroxylase (Sabban et al., 1983). Thus, the N-terminal microheterogeneity may be due to differential signal peptidase activity or due to amino peptidase activity after cleavage of the signal peptide. The role of the signal peptide is most likely to direct the enzyme to the secretory pathway and thereby ultimately to the chromaffin granules. However, intense interest and debate has centered on other potential roles for the signal peptide.

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[1] Abbreviations: LC/ESI/MS, high-performance liquid chromatography/electrospray ionization/mass spectrometry; MALDI/MS, matrix-assisted laser desorption/mass spectrometry; MS, mass spectrometry; FABMS, fast atom bombardment mass spectrometry; BPI, base peak ion; EPR, electron paramagnetic resonance; EXAFS, extended X-ray absorption fine structure; Glu-C, endoprotease Glu-C; TRIS, tris(hydroxymethyl)aminomethane; HPLC, high-performance liquid chromatography; TFA, trifluoroacetic acid; and PTH, phenylthiohydantoin.

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In the absence of any evidence for a transmembrane segment in the enzyme, Taljanidisz and co-workers proposed that the signal peptide may be the membrane anchor for membrane-bound dopamine β-hydroxylase and provided N-terminal sequencing evidence that purified membrane-bound dopamine β-hydroxylase contains the signal peptide (Taljanidisz et al., 1989). Investigations in another laboratory originally found no evidence of the signal peptide in membrane-bound enzyme (Taylor et al., 1989), but later found that 20% of the purified membrane bound form contained signal peptide (Gibson et al., 1993). However, expression of dopamine β-hydroxylase in insect cells has revealed that complete removal of the signal peptide does not result in total conversion to the soluble form (Gibson et al., 1993). It has been proposed that tight binding of phosphatidylserine to the enzyme might provide an alternative basis for membrane association (Taylor & Fleming, 1989; Gibson et al., 1993), but these results have not been confirmed by others (Bon et al., 1991). Glypiation of the enzyme does not occur (Stewart & Klinman, 1988), and the enzyme does not incorporate any acyl derivatives of palmitate (McHugh et al., 1985). Therefore, the exact functional role of the signal peptide in secretion and membrane binding has yet to be clearly understood.

Aside from the important and interesting biochemistry of the enzyme's multiple forms, dopamine β-hydroxylase also remains a potentially important target for antihypertensive therapy. Numerous inhibitors of dopamine β-hydroxylase have been synthesized, though as of yet there are no available drugs based on these compounds. However, captopril, an angiotensin-converting enzyme inhibitor, has been shown to be a reversible inhibitor of dopamine β-hydroxylase (Palatini et al., 1989). Moreover, all of the antihypertensive effects of captopril are not necessarily due to inhibition of converting enzyme (Unger et al., 1983), and it has been postulated that some of the effects may be due to dopamine β-hydroxylase inhibition (Palatini et al., 1989). Here, structural information about dopamine β-hydroxylase could contribute to drug design.

Obtaining knowledge of the disulfide linkages in dopamine β-hydroxylase would be one of the first steps in constructing a model of the enzyme. Amino acid analysis has shown that the bovine enzyme contains 14 cysteine residues, and protein and cDNA sequencing have confirmed the presence of 14 cysteines in bovine enzyme (Robertson et al., 1991; Lewis et al., 1990). Titration with 5,5′-dithiobis(2-nitrobenzoic acid) has demonstrated that none of these exist as free sulfhydryls (Ljones et al., 1976). The human enzyme contains 16 cysteines. Thus, naturation of the bovine protein *in vivo* requires the correct oxidation of 14 cysteines to seven disulfide bonds, including both intra- and intermolecular disulfide bonds. Previous studies, however, have provided no information on the actual location of disulfides in the protein. The work presented here was carried out to identify the disulfides and thus to provide the first pieces of structural information necessary to a complete structure of the enzyme.

MATERIALS AND METHODS

Reagents. Trifluoroacetic acid, iodoacetic acid, and TPCK-treated trypsin were from Sigma Chemical Co. Ultra pure guanidine–HCl and urea were from Schwartz/Mann Biotech. Acetonitrile, 2-propanol, and HPLC-grade water were from J. T. Baker. Concanavalin A-Sepharose was from Pharmacia LKB Biotechnology. [(Diethylamino)ethyl]cellulose (DE-52) was from Whatman. Catalase and endoprotease Glu-C were from Boehringer Mannheim Biochemicals. All other reagents were of the highest quality available.

Enzyme Purification. Bovine dopamine β-hydroxylase was purified from whole adrenal medulla as previously described, with several modifications (Colombo et al., 1987). Adrenal medullae were collected at a slaughterhouse, stored in 0.3 M sucrose on ice during collection, frozen in liquid nitrogen at the end of the day, and stored at −70 °C. Enzyme was prepared from ≈500 g of frozen medullae as described, with the exception that ultracentrifugation of the initial homogenate at 100000*g* was not performed. Purified enzyme was stored as a precipitate in 2 M ammonium sulfate, 25 mM KH_2PO_4, pH 6.5. Prior to protease digestion, precipitates were dissolved in buffer and dialyzed as described below. Enzyme used in these studies had specific activities of 30–50 μmol of O_2 consumed/min/mg. Amino acid positions are numbered from the N-terminus of the leader sequence as described previously (Robertson et al., 1991).

Enzyme Assay. Enzyme activity was measured by monitoring oxygen consumption polarographically with a Clark oxygen electrode at pH 5.0 and 37 °C as described previously, with the exception that the fumarate concentration was 20 mM rather than 10 mM (Colombo et al., 1987). Protein concentrations were determined spectrophotometrically at 280 nm using an extinction coefficient of $\epsilon^{1\%} = 10.8$ (Robertson et al., 1991). A tetramer mass of 290 000 Da was used in calculations of enzyme concentrations.

Trypsin Digestion. Precipitated enzyme was dissolved in 1 mL of 50 mM KH_2PO_4, pH 6.5, to a final concentration of 5–10 mg/mL. Enzyme then was dialyzed against three changes of 1 L of buffer, and the recovered enzyme was made 4 M in urea by addition of solid urea. Trypsin was dissolved to a final concentration of 1 mg/mL in 1 mM HCl, and 2% trypsin (w/w) was added to dopamine β-hydroxylase. Proteolysis was allowed to proceed for 24 h at 37 °C and was quenched by addition of 20 μL of 85% phosphoric acid. Digested enzyme either was stored at −20 °C or was analyzed by HPLC immediately. Typically, collection of peptides from a single digest required five to 10 injections. Identical peptide fractions from multiple injections were pooled for subsequent analysis. Isolated peptides were stored at 4 °C. Five separate tryptic digests were analyzed during the course of this study.

Glu-C Digestion. Precipitated enzyme was dialyzed against potassium phosphate buffer (50 mM). Endoprotease Glu-C was dissolved to a final concentration of 1 mg/mL in H_2O, and 5% Glu-C (w/w) was added to dopamine β-hydroxylase. Proteolysis was allowed to proceed for 48 h at 37 °C.

Pepsin Digestion. Pepsin was dissolved in 5% formic acid, pH 2.0, to a final concentration of 1 mg/mL. For digestion of intact dopamine β-hydroxylase, 5–10 mg of precipitated enzyme was dissolved in 1 mL of 5% formic acid and was dialyzed against three 1 L changes of 5% formic acid. Pepsin was added to dopamine β-hydroxylase in a ratio of 1:50 (w/w), and proteolysis was allowed to proceed at room temperature for 24 h. At the end of 24 h the digest was analyzed by HPLC. For a single digest, three to five injections were made to separate and collect the peptides.

For pepsin digestion of purified peptides, peptides were lyophilized to dryness and then dissolved in 0.5–1.0 mL of 5% formic acid. Two μg of pepsin was added to peptide samples, and digestion was allowed to proceed at room temperature for 24 h. After 24 h, the peptides were analyzed by HPLC. While no effort was made to determine the concentrations of peptide samples, 2 μg of pepsin was always sufficient for digestion, and no digestion products of pepsin were detectable in blank reactions.

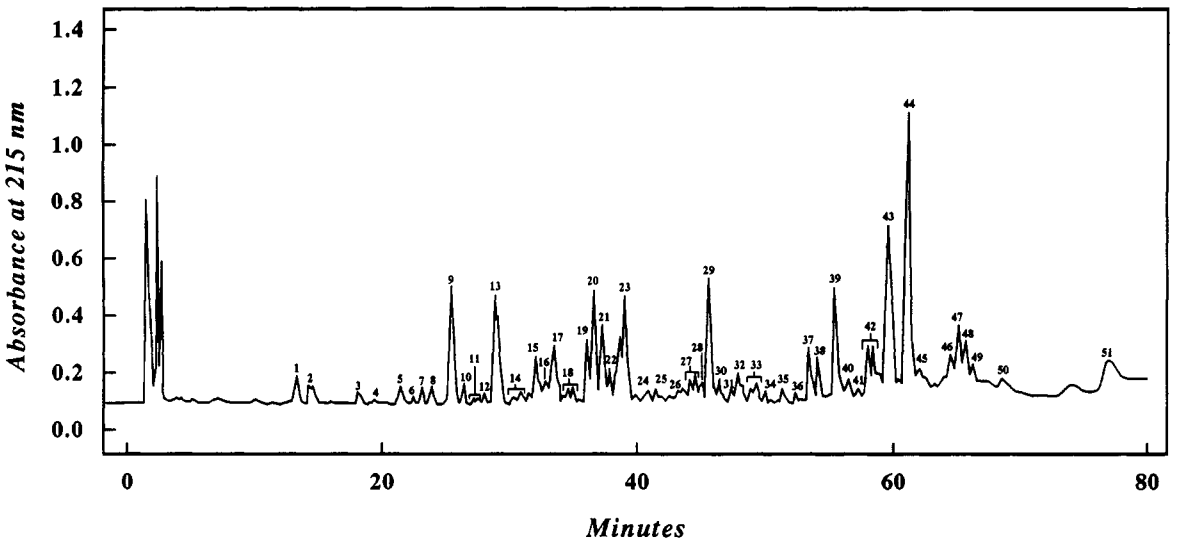

FIGURE 1: Tryptic HPLC chromatogram of native dopamine β-hydroxylase. Peptides from a 5 mg digest of dopamine β-hydroxylase were separated in multiple injections, and the corresponding fractions in each chromatogram were pooled. Aliquots of each individual pooled fraction then were lyophilized and assayed for disulfide content.

Disulfide Assays. The presence of disulfides in intact protein or in isolated peptides was quantitated as described previously (Thannhauser et al., 1984). For intact dopamine β-hydroxylase, 6 M guanidine–HCl was used in the cleavage buffer. For the data in Figure 1, 5 mg of dopamine β-hydroxylase was digested and chromatographed as described above. Individual HPLC fractions were collected and lyophilized to a volume of ≈50 μL. Disulfide cleavage buffer containing 200 mM TRIS, pH 9.5, 3 mM EDTA, 3 M guanidine–HCl, and 100 mM Na_2SO_3 was prepared, and 350 μL of cleavage buffer was added to 50 μL of peptide. Assays were started by addition of 20 μL of disodium 2-nitro-5-thiosulfobenzoate prepared as described (Thannhauser et al., 1984), and the absorbance at 412 nm was measured after 5 min at room temperature. A fresh source of disodium 2-nitro-5-thiosulfobenzoate was critical for accurate results.

Edman Sequencing. Peptides were sequenced on an Applied Biosystems Model 477A protein sequencer at the Penn State Biotechnology Center. Sequencing chemicals were from Applied Biosystems.

Reduction and (S)-Pyridylethylation. Derivatization of cysteine residues with 4-vinylpyridine was performed as described (Fullmer, 1984). Peptides were lyophilized to dryness and redissolved in 50 μL of 250 mM TRIS, pH 8.5, containing 6 M guanidine–HCl. Dithiothreitol then was added to a final concentration of 20 mM. After addition of dithiothreitol, the samples were flushed with argon, sealed in eppendorf tubes, and left for 2 h at 37 °C. After 2 h, 5 μL of 4-vinylpyridine was added, and the samples were flushed with argon and were left to react a further 30 min. At the end of 30 min, the derivatized peptides were separated by HPLC.

Peptide HPLC. Peptides were purified by reversed-phase HPLC on a Vydac C_{18} column. Solvent A contained 0.1% trifluoroacetate in water, solvent B contained 0.1% trifluoroacetate in acetonitrile, and solvent C contained 0.1% trifluoroacetate in 50:50 acetonitrile:2-propanol. Peptides from a complete digest of dopamine β-hydroxylase first were separated in a linear gradient running from 95% A–5% B to 45% A–55% B over 100 min. Individual fractions from the first chromatograph then were repurified in a linear gradient running from 95% A–5% C to 45% A–55% C over 100 min.

Microbore LC Electrospray Mass Spectrometry (LC/ESI/MS). Tryptic digests of dopamine β-hydroxylase were analyzed by microbore LC/ESI/MS using two different mobile phases: solvent system 1 containing water/acetonitrile/TFA and solvent system 2 where solvent A contained 0.1% formic acid in water and solvent B contained 0.05% formic acid in 5:2 ethanol/propanol (Feldhoff, 1991; Stacey et al., 1993; Medzihradszky et al., 1994). A dual syringe pump (Carlo Erba or Applied Biosystems) was used to deliver mobile phase at a flow rate of 50 μL/min. Microbore HPLC separations were performed on an Aquapore 300 C_{18} microbore column, 1.0 mm i.d. × 100 mm (Applied Biosystems). Fifty pmol aliquots of a dopamine β-hydroxylase tryptic digest were injected (Rheodyne, Model 8125) onto the column. The gradient for both solvent systems was started immediately after the sample injection from 2% B (water/acetonitrile) or 5% B (water/ethanol/propanol), and solvent B was linearly increased (0.75%/min) to 50% and 40%, respectively. Column effluent was monitored by a variable wavelength UV detector (Applied Biosystems) equipped with a high sensitivity capillary flow cell (LC Packings) at 215 nm. The microbore HPLC system was interfaced by a 1 M length of fused silica capillary tubing to either a VG Biotech/Fisons BIOQ or Platform mass spectrometer equipped with an electrospray source. Separations performed with the water/acetonitrile/TFA solvent system required postcolumn addition of 2-methoxyethanol/2-propanol (1:1) to optimize MS detection. To accomplish the postcolumn addition, a separate syringe pump (Isco) delivered this solvent at a flow rate of 40 μL/min to a 3.1 μL dead volume PEEK mixing tee (Upchurch Scientific) positioned after the UV detector. After the mixing tee, a zero dead volume tee was incorporated to split the column effluent 1:20 so that the flow rate into the mass spectrometer was 3–5 μL/min, while the remaining sample was manually collected for subsequent analysis. For the water/ethanol/propanol/formic acid solvent system the addition of a makeup solvent was not necessary, and optimal sensitivity was achieved without splitting the flow. Typical operating voltages for the electrospray mass spectrometers were as follows: probe tip 4200 V, counter electrode 550 V, and sampling orifice 40–50 V. The source temperature was maintained at 60 °C. The mass spectrometer was scanned in the noncontinuum mode over a range of m/z 350–2000 at 5 s/scan.

Matrix-Assisted Laser Desorption Mass Spectrometry (MALDI/MS). Microbore HPLC fractions of dopamine β-hydroxylase tryptic peptides collected from the microbore

LC/ESI/MS were concentrated to ≈10 μL. To 1 μL of this solution (containing ≈5–10 pmol of peptide) was added 1 μL of α-cyano-4-hydroxycinnamic acid (Sigma) matrix solution (50 mM, 1:2 acetonitrile:0.1% TFA in H_2O). About 1 μL of the resulting solution was spotted onto a 20-spot stainless steel sample slide. The reduced HPLC fractions were prepared by adding 2 μL of dithiothreitol (0.75 M) to 1 μL of the HPLC fraction. The reaction tube was flushed with nitrogen and left to stand for 1 h. Matrix (2 μL) was then added, and 1 μL of the mixture was spotted on the sample slide. Analysis by MALDI mass spectrometry was performed on a Kratos KOMPACT MALDI III LD/TOF mass spectrometer (Kratos Analytical) operating at 20 kV accelerating potential in the positive ion linear mode. Mass spectra were generated from the sum of 20–50 laser shots. External mass calibration was provided by the $[M + H]^+$ (m/z 1695.5 Da, average mass), $[M + 2H]^{2+}$ (m/z 8476.75), and $[M + 3H]^{3+}$ (m/z 5651.16) ions of horse heart myoglobin (Sigma).

RESULTS

Number of Disulfides in Dopamine β-Hydroxylase. Several studies have reported that there are no free sulfhydryls in dopamine β-hydroxylase and that native dopamine β-hydroxylase exists as a disulfide linked dimer (Ljones et al., 1976; Saxena et al., 1985). The absence of free sulfhydryls was verified by titrating the protein with 5,5′-dithiobis(2-nitrobenzoic acid) in the presence of 6 M guanidine–HCl and 200 mM TRIS, pH 9.5. Under these conditions, no reaction with 5,5′-dithiobis(2-nitrobenzoic acid) was detected. This result implies that all 14 cysteines in dopamine β-hydroxylase are involved in disulfide bonds.

The actual number of disulfide bonds was measured in the presence of sodium sulfite and disodium 2-nitro-5-thiosulfobenzoate as described previously (Thannhauser et al., 1984). An average of six determinations yielded a value of 6.7 ± 0.4 disulfides/monomer dopamine β-hydroxylase. The error corresponds to ±6% of the total value and is close to the reported limits of ±3% in the assay (Thannhauser et al., 1984). Considering the error in the measurement, the value of 6.7 disulfides/monomer may actually represent seven disulfides/monomer, which would account for all 14 cysteines.

Identification of Disulfide-Containing Peptides in Dopamine β-Hydroxylase. Peptide maps of native dopamine β-hydroxylase were generated by reversed-phase HPLC after digestion with trypsin, endoprotease Glu-C, and pepsin, respectively. Disulfide-containing fractions in each of the peptide maps were identified by assaying aliquots of each fraction as described in the Materials and Methods (Thannhauser et al., 1984). After disulfide-containing fractions were identified, the material in each of these fractions was rechromatographed in a different solvent system to obtain chromatographically pure peptides. One aliquot of the pure peptide was sequenced by Edman degradation. The remaining sample was reduced and (*S*)-pyridylethylated and then was rechromatographed to obtain the individual peptides arising from reduction of the disulfide linked species.

Figure 1 illustrates the peptide profile of a typical tryptic digest of dopamine β-hydroxylase. Similar profiles were obtained on five separate digests. Fifty-one fractions were collected, and disulfide analysis indicated that fractions 38, 39, and 42–51 contained significant amounts of disulfide. These fractions were collected from multiple chromatographs and were analyzed individually.

In addition, pepsin also was used to digest intact dopamine β-hydroxylase, and the proteolytic products from pepsin digests

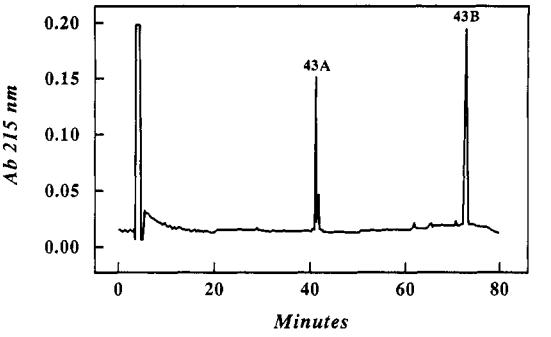

FIGURE 2: Purification of peptides in fraction 43. Fraction 43 from the tryptic digest in Figure 1 was rechromatographed after reduction and (*S*)-pyridylethylation as described in the Materials and Methods.

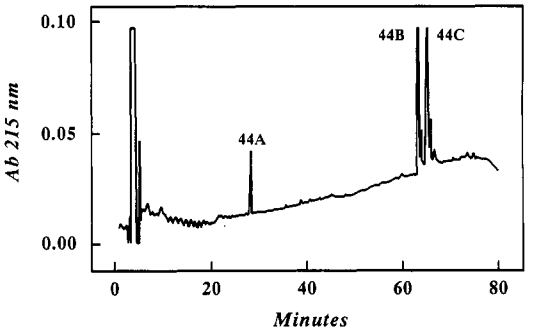

FIGURE 3: Purification of peptides in fraction 44. Fraction 44 from the tryptic digest in Figure 1 was rechromatographed after reduction and (*S*)-pyridylethylation as described in the Materials and Methods.

were separated in the same way as the data shown in Figure 1. In the case of pepsin, however, 70 peptide fractions were collected and at least 15 fractions demonstrated significant amounts of disulfide. The higher number of disulfide-containing fractions indicates that pepsin generated different length peptides containing the same disulfide. Several contiguous fractions containing the highest amounts of disulfide were pooled and sequenced.

Analysis of Disulfide-Containing Peptides by Edman Sequencing. Pair 1: Cys140–Cys582. Fraction 43 from Figure 1 was rechromatographed under different solvent conditions to assess its purity. The sample eluted as a single fraction and therefore appeared to contain a single peptide (data not shown). After reduction and (*S*)-pyridylethylation, the sample migrated as two fractions, as shown in Figure 2. These two fractions were collected and sequenced. However, for peptide 43B, Edman degradation did not extend to the end of the peptide, and the C-terminal glycine residue (Gly603) was not detected. The sequences of fractions 43A and 43B were as shown below. Peptides 43A and 43B each contained a single cysteine. This result permits assignment of a disulfide between Cys140 and Cys582.

peptide 43A	136-PFGTCDPNDYLIEDGTVHLVYGFLEEPLR-164
peptide 43B	575-LEEPTPHCPASQAQSPAGPTVLNISGGK-602

Pairs 2 and 3: Cys218–Cys269 and Cys255–Cys281. Fraction 44 from Figure 1 was rechromatographed and also appeared to contain a single peptide (data not shown). After reduction and (*S*)-pyridylethylation, chromatography yielded three major fractions, as shown in Figure 3. The peptides in these fractions contained the sequences shown in Chart 1. Peptide 44B contained two cysteines, while peptides 44A and 44C each contained one cysteine. From these data, the disulfide linkages could not be determined. However, cleavage of native dopamine β-hydroxylase with pepsin produced several

Chart 1

peptide 44A 278-LNF **|CR**-282

peptide 44B 230-HHIVMYEPIVTEGNEALVHHMEV **|FQCAAEF|ETIPHFSGPCDSK**-272

peptide 44C 204-APDVLIPGQQ **|TTYWCY|** VTELPDGFPR-229

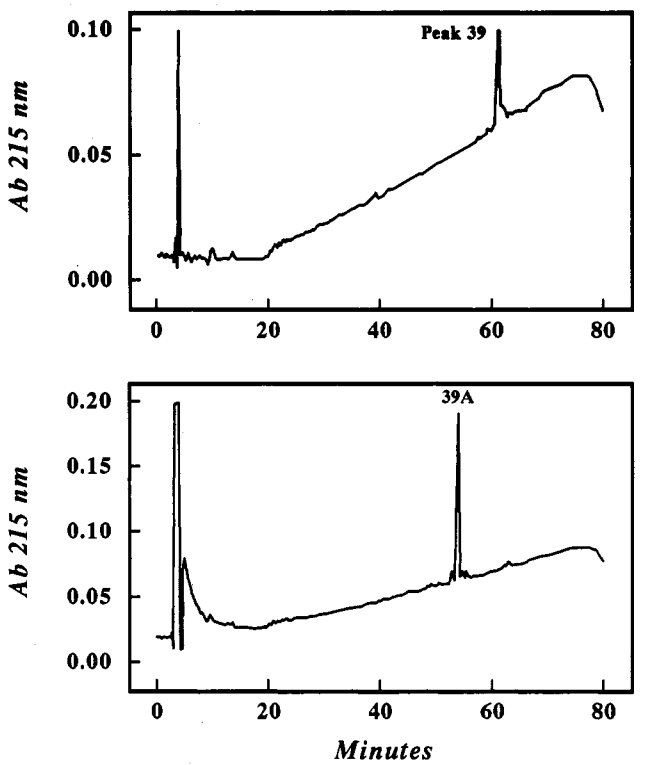

FIGURE 4: Purification of peptides in fraction 39. Fraction 39 from the tryptic digest in Figure 1 was rechromatographed as described in the Materials and Methods. The upper panel represents the native form of fraction 39, and the lower panel represents the reduced form.

products containing the disulfide linkages shown in Chart 1, where the bold amino acids and vertical bars indicate the peptides obtained by pepsin cleavage. From Edman sequencing of the peptic and tryptic peptides, disulfides Cys218–Cys269 and Cys255–Cys281 can be assigned.

Pairs 4 and 5: Cys514–Cys514 and Cys516–Cys516. Fraction 39 from Figure 1 was rechromatographed to assess its purity, and Figure 4, top panel, shows that the sample appeared to contain a single peptide. After reduction and (*S*)-pyridylethylation, the sample migrated as single fraction with a different retention time, as shown in Figure 4, bottom panel. Sequencing of fraction 39A showed that it contained a single peptide which included the sequence 514-Cys-Thr-Cys-516. These data indicated either that there was an intramolecular disulfide between Cys514 and Cys516 or that there were two intermolecular disulfides between two identical peptides. In order to assign these disulfides as intra- or intermolecular, we looked for evidence of PTH–cystine in sequencing cycles 7 and 9.

Previous workers have reported that PTH–cystine can be detected as a small peak eluting after PTH–tyrosine on an Applied Biosystems gas phase sequencer (Højrup & Magnusson, 1987). In the sequencing analysis of native fraction 39, we observed two small peaks eluting after PTH–tyrosine in the HPLC separation of PTH amino acids from sequencing cycles 7 and 9. These corresponded to PTH cystine. While the yields were low, we have used this evidence to conclude that Cys514 pairs with Cys514 in an intermolecular disulfide. Similarly, we have concluded that Cys516 and Cys516 form an intermolecular disulfide. We considered the possibility that Cys514 pairs with Cys516 as an intermolecular disulfide, but this arrangement would give a blank in the sequencing data when the cycle containing Cys514 was derivatized. Cycle 7 was not blank and clearly contained evidence of PTH cystine. On the basis of this result, two intermolecular disulfides between subunits can be assigned at positions 514 and 516. This peptide was analyzed further by mass spectrometry, as described below.

sequencing cycle .....7 .9.....

subunit A peptide 39A 508-FNSEEVCTCPQASVPEQFASVPWNSFNR-535

subunit B peptide 39A 508-FNSEEVCTCPQASVPEQFASVPWNSFNR-535

Pair 6: Cys452–Cys474. Traditional chemical methods were able to identify disulfide bonds involving eight cysteines. By inference, the remaining six cysteines, Cys452, Cys474, Cys376, Cys489, Cys380, and Cys551, were known to make the last three disulfide linkages, but these peptides could not be isolated in pure form. Fraction 46 from Figure 1 was sequenced by Edman degradation and was found to contain at least five separate sequences, which could only be identified by careful analysis of the data and by reference to the known sequence of the protein. This mixture of peptides contained all six of the remaining cysteines. Fractions 47–49 also contained the same mixture of peptides. Fractions 46–49 apparently compose a tightly disulfide cross-linked trypsin resistant core in dopamine β-hydroxylase. Fractions 46–49 were pooled and then subjected to pepsin digestion, which produced more than 30 fractions in the peptide map. A significant amount of disulfide was detected and recovered in fraction 22 (data not shown). The material was sequenced directly without reduction and purification of the individual fragments, and contained the following sequences,

449-ITSCTYNTEDRRLA-462

471-EEMCVNYVHYYPQTQ-485

The presence of only one cysteine on each peptide permits assignment of a disulfide linkage between Cys452 and Cys474. The remaining two disulfide bonds could not be identified in any discrete peptide in the pepsin digest. Consequently, mass spectrometry was employed to obtain a complete analysis of the disulfides and thereby to identify the linkages that could not be obtained by chemical sequencing methods.

Analysis of Disulfide-Containing Peptides by Mass Spectrometry. Tryptic and endoprotease Glu-C digests of dopamine β-hydroxylase were analyzed by microbore LC/ESI/MS. Figure 5a shows the UV absorbance chromatogram of the tryptic digest, and Figure 5b shows the base peak ion (BPI) chromatogram of the same digest. Figure 6 shows the BPI chromatogram of the Glu-C digest. Each of the fractions in the BPI chromatograms are labeled according to the corresponding tryptic or Glu-C peptide(s) detected in that fraction. The observed masses of the tryptic and Glu-C peptides in each case were generally within 0.5 Da of the expected mass.

Analysis of the dopamine β-hydroxylase tryptic digest by LC/ESI/MS confirmed 84% of the primary sequence. Three fractions eluting at 44.8, 49.1, and 51.4 min, respectively (fractions A–C in Figure 5b), contained ions whose molecular

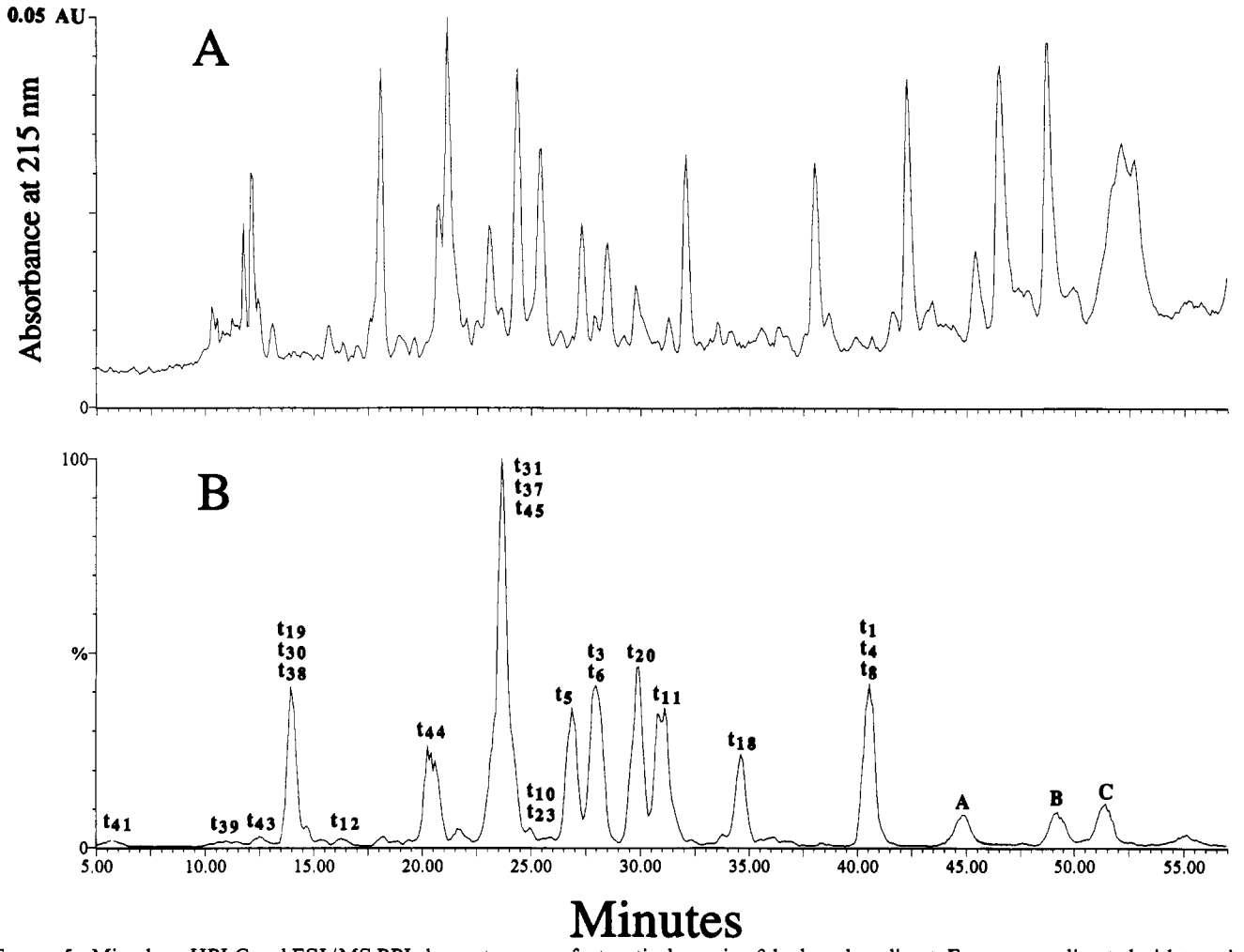

FIGURE 5: Microbore HPLC and ESI/MS BPI chromatograms of a tryptic dopamine β-hydroxylase digest. Enzyme was digested with trypsin and was analyzed by LC/ESI/MS as described in the Materials and Methods. Part a shows the HPLC UV trace of the peptide separation, and part b shows the BPI chromatogram as measured by the electrospray mass spectrometer. The chromatographic separation utilized the water/acetonitrile/TFA solvent system. The BPI chromatogram is offset by a 90 s delay between the HPLC and mass spectrometer. Tryptic peptides, as analyzed by the mass spectrometer, are numbered from the N-terminus, where t_1 is the N-terminal peptide starting at Ala29. Fractions that contained molecular masses consistent with disulfide linked peptides are labeled A–C. The masses determined for these fractions are listed in Table 1. See Figure 10 for a summary of the masses of all the tryptic peptides observed in the LC/ESI/MS analysis.

masses could not be assigned to simple tryptic peptides. The fraction at 44.8 min contained a peptide of mass 6345.5. The fraction eluting at 49.1 min contained three peptides of masses 4880.2, 6253.0, and 6310.6. The fraction at 51.4 min contained a peptide of mass 8497.9. Figure 7a–c show the ESI spectra of these three fractions, respectively. These masses can be assigned to disulfide-linked peptides.

Mass 6345.5 (ion series A, calculated mass 6345.9, Figure 7a) corresponds to a disulfide-linked peptide with intermolecular disulfides at Cys514 and Cys516. These data confirm the presence of intermolecular disulfides detected by chemical sequencing of fraction 39 from Figure 1. Mass 4880.2 (ion series B, calculated mass 4880.69, Figure 7b) corresponds to a peptide with an intramolecular disulfide linkage between Cys380 and Cys551. These data identify a linkage that was not obtained by chemical sequencing. Masses 6253.0 and 6310.6 (ion series C, calculated masses 6252.98, and ion series D, 6310.03, Figure 7b) correspond to a peptide containing a disulfide linkage between Cys140 and Cys582, where the mass difference between the two peptide masses is evidence of incomplete cleavage of the C-terminal glycine residue. This result demonstrates that the dopamine β-hydroxylase polypeptide chain terminates at Gly603, which was not demonstrated by chemical sequencing of peptide 43B.

Mass 8497.9 (ion series E, calculated mass 8497.66, Figure 7c) corresponds to a peptide fragment containing two disulfide linkages between three individual peptides. The three individual peptides are T_{13}, T_{14}, and T_{16}. Peptide T_{14} (amino acids 230–273) contains Cys255 and Cys269. Peptide T_{13} (amino acids 204–272) contains Cys218, and peptide T_{16} (amino acids 277–282) contains Cys281. The arrangement of cysteines on these peptides indicates that peptides T_{13} and T_{16} are connected to peptide T_{14}. This result confirmed the chemical sequencing of peptides 44A, 44B, and 44C. The individual disulfide linkages among these three peptides were determined from the Glu-C digest, as described below.

The dopamine β-hydroxylase tryptic digest was also analyzed by LC/ESI/MS using an alternative mobile phase composed of water/ethanol/propanol/formic acid. This mobile phase altered the elution order of tryptic peptides and thus provided complimentary information to the original analysis (Feldhoff, 1991; Stacey et al., 1993; Medzihradszky et al., 1994). Figure 8a,b shows partial BPI chromatograms depicting the elution profiles of the most hydrophobic peptides (retention times >38 min) in each solvent system. Comparison of the ESI spectra shown in Figure 7b,d illustrates the advantage of using the nontraditional solvent system.

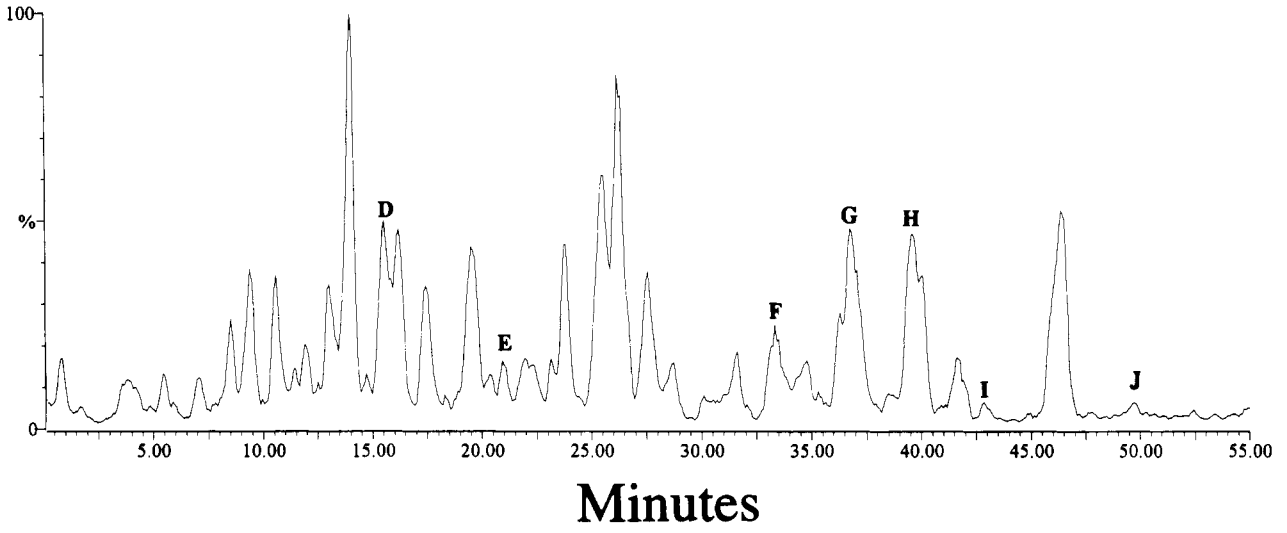

FIGURE 6: Microbore LC/ESI/MS BPI chromatogram of an endoprotease Glu-C dopamine β-hydroxylase digest. Enzyme was digested with Glu-C and was analyzed by LC/ESI/MS as described in the Materials and Methods. Shown is the BPI chromatogram from a microbore LC/ESI/MS analysis (UV chromatogram not shown) as measured by the electrospray mass spectrometer. Fractions that contained molecular masses consistent with disulfide-linked peptides are labeled D–J. The masses determined in these fractions are listed in Table 1. Figure 10 summarizes the masses of all the Glu-C peptides obtained from the LC/ESI/MS analysis.

The disulfide-linked peptide with a molecular mass 4880.2 Da [represented by the multiply charged ion series B at m/z 977.0 (+5) and m/z 1220.5 (+4)] seems to be a relatively minor component in Figure 7b. When analyzed using the alternative solvent system this peptide is chromatographically resolved from the other components (cf. fraction eluting at 50.0 min, Figure 8b). This resulted in a better signal to noise ratio in the ESI spectrum, as shown in Figure 7d, and provided convincing evidence for the disulfide linkage between Cys380 and Cys551. It was important to obtain good quality ESI data because the Cys380–Cys551 linkage was not identified by chemical peptide sequencing and the assignment rests solely on this spectrum.

The relatively small signal derived from the peptide containing the latter Cys380–Cys551 linkage, as compared to the other disulfide-linked peptides, may be due to partial glycosylation at Asn552 (Robertson et al., 1990). If each Asn552 site in the protein preparation were glycosylated, the native peptide with a molecular mass 4880.2 Da, due solely to the mass of amino acids, would not be observed. The fact that it is observed indicates that not all of the Asn552 sites are glycosylated. However, the low abundance implies that only a small proportion of the Asn552 sites are free, and in fact the preponderance of sites are glycosylated.

The fractions containing disulfide-linked peptides in the tryptic digest separated under standard water/acetonitrile/trifluoroacetic acid chromatographic conditions (Figure 5b, fractions A–C) were further analyzed by MALDI/MS. Figure 9a–c illustrates the MALDI spectra of the three fractions, whereas Figure 9d–f illustrates the MALDI spectra of each corresponding fraction after reduction with dithiothreitol. The molecular masses of the five disulfide-linked peptides detected by MALDI/MS are in agreement with the masses observed in the electrospray spectra (Table 1). Figure 9a illustrates the MALDI spectrum of the native peptide with molecular mass 6345.6, which corresponds to the dimer of peptide 39A identified by chemical sequencing. Figure 9d shows the MALDI spectrum of the reduced peptide, which yielded a mass of 3175.8, as would be expected for peptide 39A if it forms a homodimer of identical peptides from separate subunits. In combination with the chemical sequencing data that provided evidence of PTH–cystine at Cys514 and Cys516 (described above), the LC/ESI/MS and MALDI/MS data confirm that an intersubunit disulfide linkage occurs at these positions.

The MALDI spectrum of the fraction in Figure 9b contains three peptides with molecular masses 4880.5, 6253.2, and 6310.8. Mass 4880.5 corresponds to native peptide $T_{26,27}$–$T_{42,43}$, which contained the Cys380–Cys551 linkage. Mass 6253.2 corresponds to native peptide T_9–T_{46}, containing the Cys140–Cys582 linkage. Mass 6310.8 corresponds to native peptide T_9–$T_{46,47}$, also containing the Cys140–Cys582 linkage. After reduction of this fraction, three peptides with masses 2787.9, 2845.6, and 3467.2 were observed, as shown in Figure 9e. The reduced peptides correspond to T_{46} (calculated molecular mass 2788.1), $T_{46,47}$ (calculated molecular mass 2845.2), and T_9 (calculated molecular mass 3467.9). The reduced peptide masses confirm the assignment of the disulfide linkage between Cys140–Cys582. The reduced components of the disulfide-linked peptide of molecular mass 4880.5 (containing disulfide linkage Cys380–Cys551) were not detected, presumably due to suppresion by the other components.

Finally, Figure 9c shows the MALDI spectrum of the peptide with molecular mass 8497.1, which contains the disulfide linkages Cys218–Cys269 and Cys255–Cys281 (peptide sequences T_{13}–T_{14} and T_{14}–T_{16}). After reduction with dithiothreitol, the MALDI spectrum demonstrated the presence of two peptides of molecular mass 2969.1, corresponding to peptide T_{13} (calculated molecular mass 2968.4, containing Cys218) and molecular mass 4881.9, corresponding to peptide T_{13} (calculated molecular mass 4882.5, containing Cys255 and Cys269), as shown in Figure 9f. The peptides of molecular masses 2969.1 and 4881.9 correspond to peptides 44A and 44B identified by chemical sequencing (Figure 3). The remaining reduced peptide, T_{16} (calculated molecular mass 652.8 containing Cys281), was not detected in the MALDI mass spectrum. However, this peptide was isolated and identified by chemical sequencing of peptide 44A (Figure 3).

The combined analysis of the tryptic digest by LC/ESI/MS and MALDI failed to identify a fragment containing a disulfide between Cys376 and Cys489. In order to demonstrate that the remaining two cysteine residues are disulfide linked, and to confirm the results obtained from the tryptic digests,

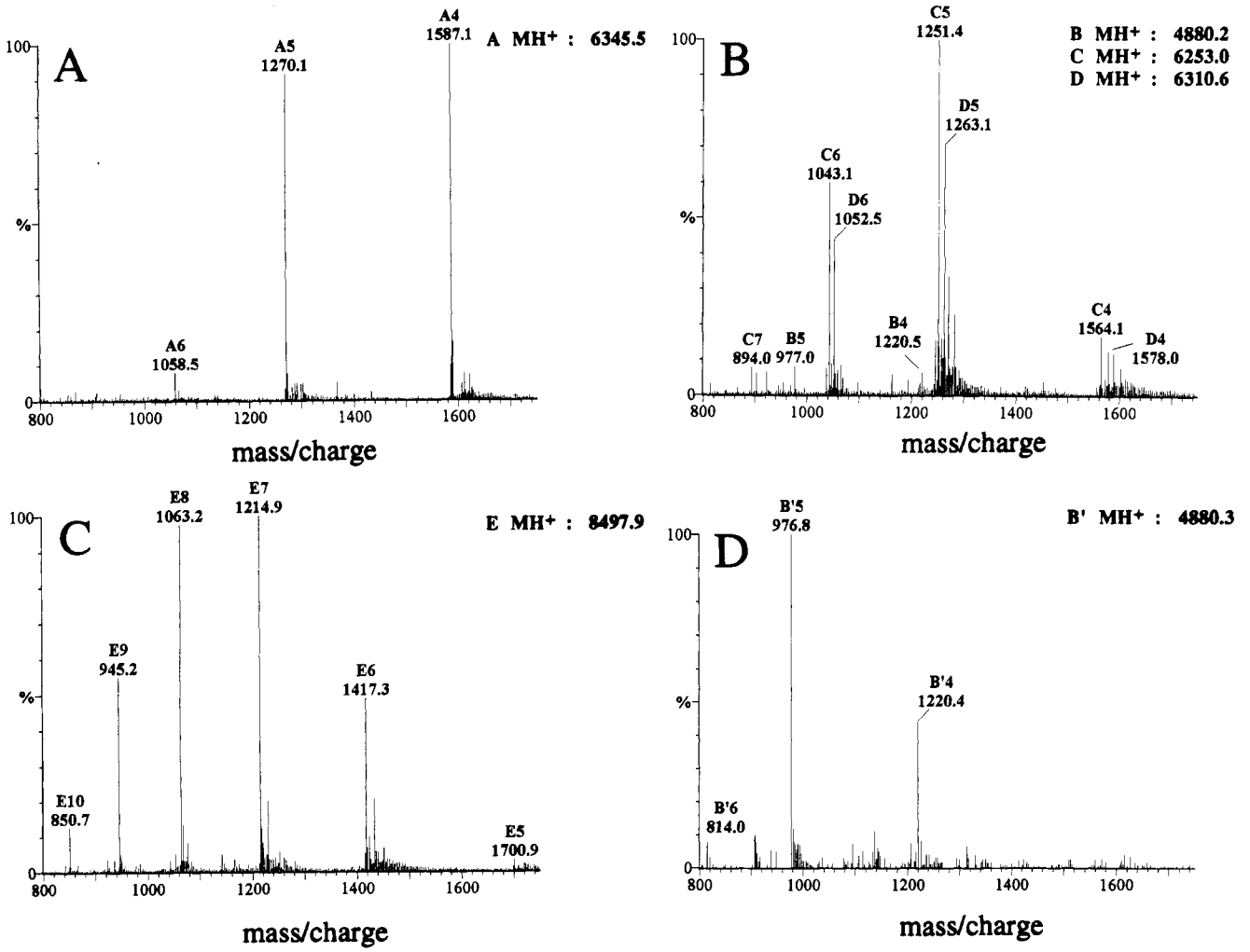

FIGURE 7: Electrospray mass spectra of tryptic peptides containing disulfide linkages. The electrospray mass spectra of native peptides eluting at 44.8, 49.1, and 51.4 min in the BPI chromatogram of Figure 5b are shown in parts a–c. These peptides were chromatographed in an acetonitrile/TFA-based solvent system, as described in the Materials and Methods. Part d shows the electrospray mass spectrum of the native peptide eluting at 50.0 min in the BPI chromatogram of Figure 8b. This peptide was chromatographed in an alcohol/formic acid solvent system, as described in the Materials and Methods. Comparison of parts b and d illustrates the increased relative intensity of the ion series representing the disulfide-linked peptide with molecular mass 4880.3 (ion series B in part b, ion series B′ in part d) as a result of a better peptide separation.

dopamine β-hydroxylase was digested with endoprotease Glu-C. Furthermore, this enzyme was chosen in order to generate subfragments of peptides T_{13}, T_{14}, and T_{16}, and thereby to confirm the assigments based on the peptic peptides described above. Figure 6 shows the BPI chromatogram of this digest, which was considerably more complex than the tryptic digest, due mainly to the large number of possible cleavage sites available to the protease (24 aspartic acid residues and 40 glutamic acid residues). Seven fractions containing multiply charged ion series that corresponded to disulfide linked peptides were identified in the Glu-C digest. These are labeled D–J in Figure 6, and Table 1 lists the molecular masses observed in these fractions.

The data in Table 1 provide evidence for a disulfide linkage between Cys376–Cys489, the one remaining linkage not identified by chemical sequencing or mass analysis of tryptic peptides. In addition, digestion with Glu-C produced the expected individual peptide fragments corresponding to the Cys218–Cys269 linkage and the Cys255–Cys281 linkage, which were predicted from chemical sequencing of peptic peptides. In summary, digestion with Glu-C established or confirmed all the disulfide linkages in the protein except the Cys380–Cys551 linkage, which was determined by analysis of the tryptic peptides.

Preliminary LC/ESI/MS experiments confirmed the presence of the oligosaccharide structures previously proposed from carbohydrate analysis (Margolis et al., 1984). The data indicate that Asn170 and Asn552 are linked to an array of complex biantennary and complex bisecting biantennary oligosaccharides, and that Asn50 is linked to high mannose oligosaccharides (data not shown).

DISCUSSION

The disulfide linkages in dopamine β-hydroxylase have been determined by a combination of peptide sequencing, LC/ESI/MS, and MALDI mass spectrometry. All but two of the disulfide linkages were confirmed by sequencing either tryptic peptides or peptides derived from pepsin digestion at pH 2.0. Several of the disulfide linked peptides identified by chemical sequencing were obtained as both trypsin and pepsin fragments, and the same disulfide linkages were observed in both cases (data not shown). This demonstrated that there was no disulfide scrambling under the digestion conditions. Mass spectrometric analysis confirmed the disulfide linkages identified by Edman sequencing and the remaining two linkages were determined solely from mass spectrometric analysis.

From the accumulated molecular weight data obtained from analysis of tryptic and Glu-C digests of dopamine β-hydroxy-

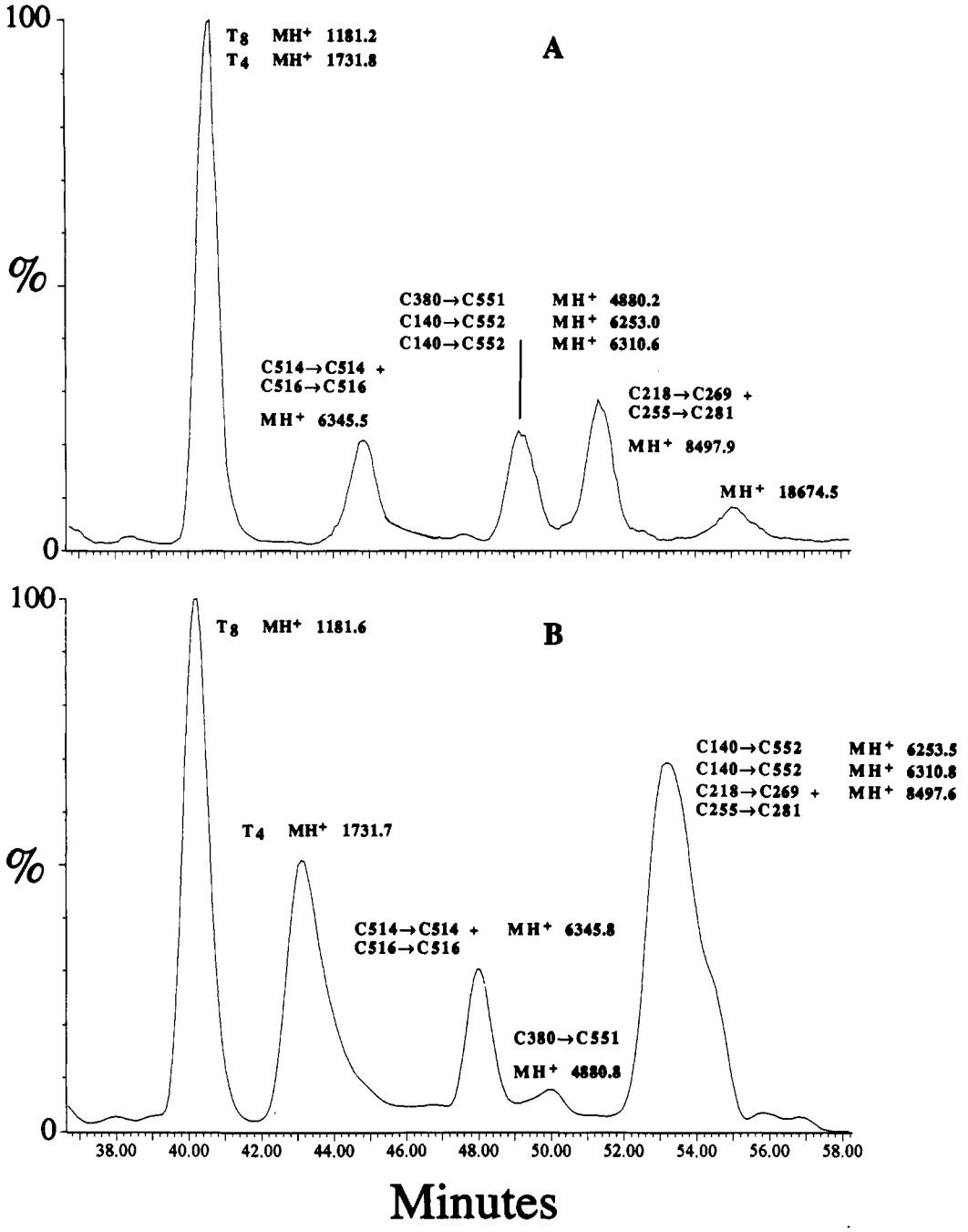

FIGURE 8: Partial BPI chromatogram of hydrophobic tryptic peptides. Tryptic peptides were separated in an acetonitrile/TFA-based solvent system or an alcohol/formic acid based solvent system as described in the Materials and Methods. Part a shows the partial BPI map of peptides eluting after 38 min in water/acetonitrile/TFA, and part b shows the partial BPI map of the same peptides eluting in H_2O/EtOH/PrOH/HCOOH. The alternative mobile phase yielded a chromatographically resolved peptide with a molecular mass of 4880.3 eluting at 50.0 min, as compared to the mixture of peptides in part a eluting at 49.1 min.

lase by LC/ESI/MS, 96% of the primary sequence was confirmed. Edman sequencing has previously demonstrated microheterogeneity at the N-terminal amino acid of mature soluble dopamine β-hydroxylase (Robertson et al., 1990; Wang et al., 1990; Taylor et al., 1989). The N-terminus detected in the tryptic digest for these samples was shown to begin at Ala29, rather than at Ser26 (Robertson et al., 1990). This is consistent with other evidence that the enzyme has a ragged N-terminus and may indicate that the peptide beginning at Ser26 was in low abundance and was not detected in these experiments. The presence of the C-terminal glycine residue, previously not detected by Edman sequencing (Robertson et al., 1990) or mass spectrometric mapping (Wang et al., 1990) was confirmed by LC/ESI/MS. Figure 10 summarizes the molecular weight fragments observed by LC/ESI/MS and illustrates their positions in the primary sequence. Figure 11 proposes a model for the topology of the protein based on the disulfide linkages and other previously determined physical data.

Analysis of the disulfide linkages revealed that there are two intermolecular disulfide linkages covalently linking a dopamine β-hydroxylase dimer. The interchain disulfides at positions 514 and 516 must represent an intermonomer subunit link because the mass of the native fragment containing these cysteines, as determined by both electrospray and MALDI mass spectrometry, was 6345.6, which can only be rationalized as a dimer of peptide 39B (tryptic peptide T_{40} in the LC/ESI/MS analysis). Furthermore, reduction of this native

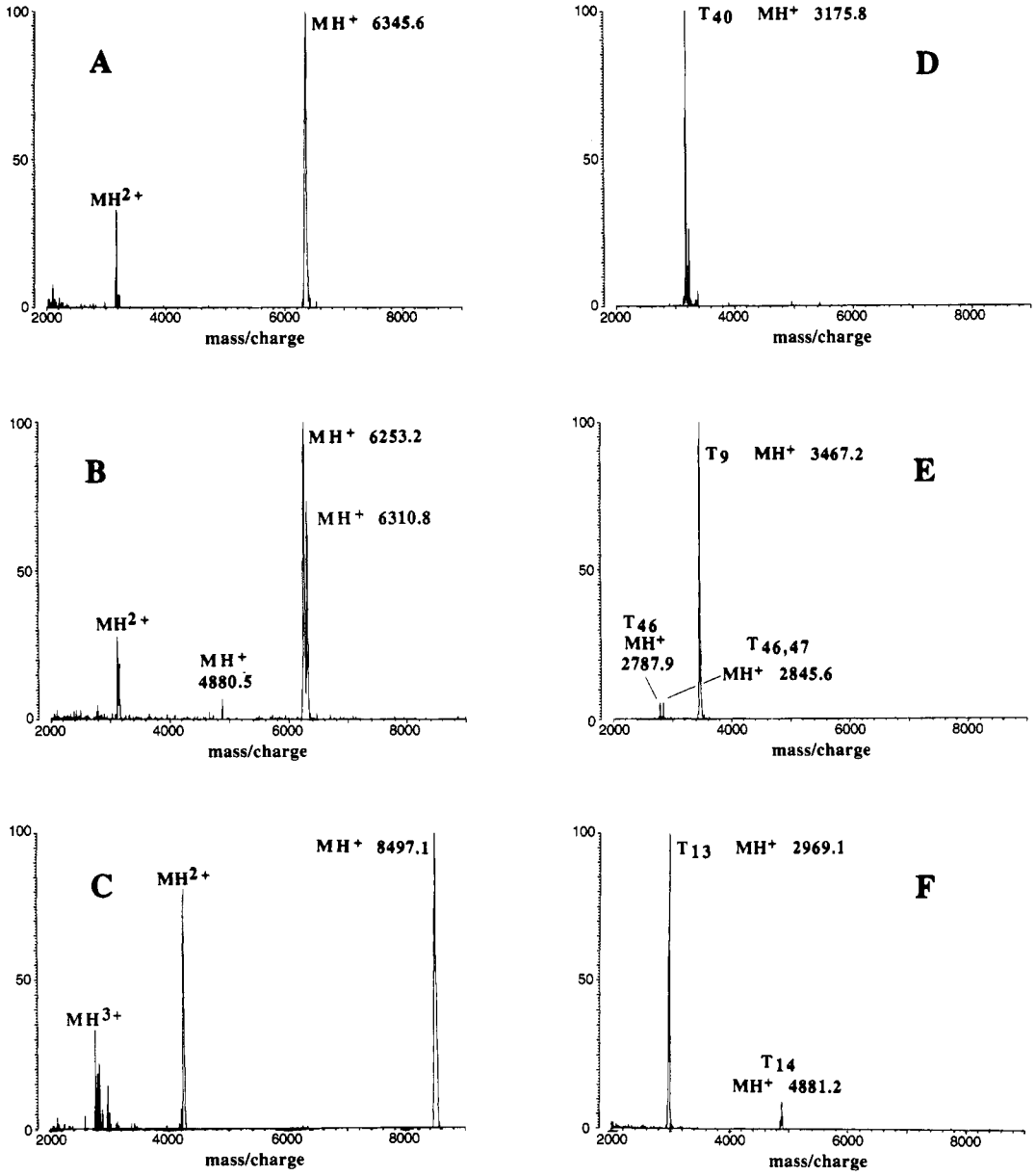

FIGURE 9: MALDI-TOF mass spectra of HPLC fractions collected from microbore LC/ESI/MS analysis (cf. fractions A–C in Figure 5b). Parts a–c illustrate the MALDI spectra of the native disulfide-linked peptide, and d–f illustrate the same fractions after reduction with dithiothreitol. Part a shows the MALDI/MS spectrum of the native peptide of molecular mass 6345.6 (disulfide linkage Cys514–Cys514 and Cys516–Cys516), which corresponds to peptide 39 identified by chemical sequencing (peptide T_{40} in the LC/ESI/MS analysis). Part d shows the MALDI/MS spectrum of the same peptide after reduction. Part b shows the spectrum for the three peptides with molecular masses 4880.5 (disulfide linkage Cys380–Cys551), 6253.2, and 6310.8 (disulfide linkage Cys140–Cys552) which correspond to peptides 43A and 43B identified by chemical sequencing. Part e shows the peptides after reduction. Part c shows the spectrum of the peptide of molecular mass 8497.1, which contained disulfides at Cys218–Cys269 and Cys255–Cys281 and corresponds to peptides 44A–C. Part f shows the reduced forms of these peptides.

fragment produced a single peptide of molecular mass 3175.8. This native fragment was confirmed in three separate trypsin digests by mass spectrometry. No other fragments were obtained as homodimers, and therefore the 514 and 516 linkages are the only unambiguous intersubunit disulfide linkages.

Each of the other linkages shown in Figure 11 might represent an intersubunit disulfide. When two different peptide fragments have been isolated as disulfide linked, there is, in principle, no way of knowing for sure if they are from the same subunit. However, we can rationalize the proposed model in Figure 11 by several arguments.

First, an intersubunit disulfide must be on the surface of the enzyme, and if any of the other disulfides shown in Figure 11 are intersubunit, they would have to be on the surface. The first candidate is the Cys140–Cys582 linkage. The N- and C-terminal regions of the protein contain the sites of glycosylation and therefore would be solvent exposed. However, tabulation of known disulfide linkages from the protein crystallography database has shown that there is a significant population of N- to C-terminal linkages that are not favored by chance (Thornton, 1981). This has been interpreted to mean that an intrachain N- to C-terminal linkage may be a favorable way of stabilizing folded structures. We take this as support for the idea that dopamine β-hydroxylase probably contains an intramolecular N- to C-terminal disulfide linkage.

The three disulfides containing cysteines 376, 380, 452, 474, 489, and 551 all were contained in a hydrophobic, protease resistant, multiply-linked segment of the protein, which eluted as fractions 46–49 in Figure 1. On the basis of protease

Table 1: Disulfide-Linked Peptides Detected in Tryptic and Endoprotease Glu-C Digests of Dopamine β-Hydroxylase Analyzed by LC/ESI/MS

peak	t_R (min)	cysteine linkage	peptide amino acids	MH^+ obsd	MH^+ calcd[a]
			Tryptic Digest		
A	44.8	C514→C514 + C516→C516	(508–535) + (508–535)	6345.5	6345.94
B	49.1	C380→C551	(380–405) + (540–558)	4880.2[b]	4880.69
		C140→C582	(135–164) + (572–602)	6253.0	6252.98
		C140→C582	(135–164) + (572–603)	6310.6	6310.03
C	51.4	C218→C269 + C255→C281	(204–229) + (230–272) and (230–272) + (278–282)	8497.9	8497.66
			Endoprotease Glu-C Digest		
D	15.5	C514→C514 + C516→C516	(513–523) + (513–523)	2263.8	2263.60
E	22.1	C376→C489	(368–378) + (488–494)	1924.9	1924.21
F	33.2	C452→C474	(447–457) + (473–487)	3129.8	3130.55
G	36.3	C218→C269	(207–222) + (261–270)	2973.2	2973.36
		C452→C474	(433–457) + (473–487)	4681.9	4682.47
H	38.4	C218→C269	(202–222) + (261–270)	3525.7	3525.99
I	42.7	C140→C582	(128–144) + (578–603)	4427.5	4428.01
J	49.6	C218→C269 + C255→C281	(202–222) + (261–299) and (252–258) + (261–299)	7669.6	7669.88
		C218→C269 + C255→C281	(202–222) + (261–300) and (252–258) + (261–300)	7798.9	7798.99

[a] Expected mass calculated from average molecular weights. [b] Nonglycosylated form.

FIGURE 10: Composite primary sequence map of dopamine β-hydroxylase. Tryptic or endoprotease Glu-C generated peptides detected by LC/ESI/MS are shown relative to the complete sequence of bovine dopamine β-hydroxylase. Peptides labeled "t" correspond to tryptic peptides, and peptides labeled "g" indicate Glu-C peptides. The numbers adjacent to the peptide designations refer to the chemical (weighted average of stable isotopes) molecular weight of the peptide observed by LC/ESI/MS. Cysteine residues and disulfide-linked peptides are indicated in bold. Molecular masses of peptides containing Asn50, Asn170, and Asn552 reflect glycosylation of these residues.

resistance of this fragment and its hydrophobic elution position, it seems likely that these disulfides form a hydrophobic domain on the interior of the enzyme and therefore would not be candidates for surface intersubunit disulfides. In addition, this group of disulfides shares some proximity to known sites of modification by mechanism-based inhibitors and therefore may be part of the active site of the enzyme.

Similarly, the two disulfides containing cysteines 218, 255, 269, and 281 follow motifs in other proteins where there is a cluster of short range disulfides (Thornton, 1981). Given the proximity to two other known sites of modification by mechanism-based inhibitors, these two short range disulfides may also be part of the active site of the enzyme. If the first cluster of three disulfides (cysteines 376, 380, 452, 474, 489, and 551) forms a hydrophobic core and part of the active site and the second group of two disulfides (cysteines 218, 255, 269, and 281) is part of the active site, it might be rationalized that the second group is also located on the interior of the protein. While this is only speculative, it seems reasonable to consider these two disulfides as interior and the Cys514–Cys514, Cys516–Cys516 pairs as the only intersubunit disulfides.

Analysis of the location of histidine residues in the protein reveals two clusters of histidines overlapping the regions of internal disulfides, as illustrated in Figure 11. The enzyme has been shown to require two Cu^{2+} atoms per monomer for maximal activity (Ash et al., 1984; Klinman et al., 1984). Spin echo EPR (McCracken et al., 1988) and EXAFS (Blumberg et al., 1989) experiments have shown that each Cu^{2+} atom has three to four histidine ligands. Each subunit would therefore be expected to have six to eight histidines in the active site to coordinate the Cu^{2+} atoms. We suggest that the coincidence of two clusters of internal disulfides and two clusters of histidine rich regions may define two Cu^{2+} binding domains that together form the active site of the enzyme.

The overall topology of an enzyme monomer, based on the model in Figure 11, would link the N- and C-terminal regions on one solvent accessible face of the protein that would also contain the known glycosylation sites. A signal peptide, if attached to the protein, would have to share some proximity to the carbohydrate-containing face of the protein by virture of being at the N-terminus. This might indicate that both the carbohydrate and signal peptide are involved in membrane association of membrane-bound dopamine β-hydroxylase, which in turn might have implications for secretion and cellular location of dopamine β-hydroxylase.

For instance, there appears to be more than one secretory pathway for dopamine β-hydroxylase. In addition to localization in the chromaffin granule, soluble dopamine β-hydroxylase has been found in the peripheral circulation.

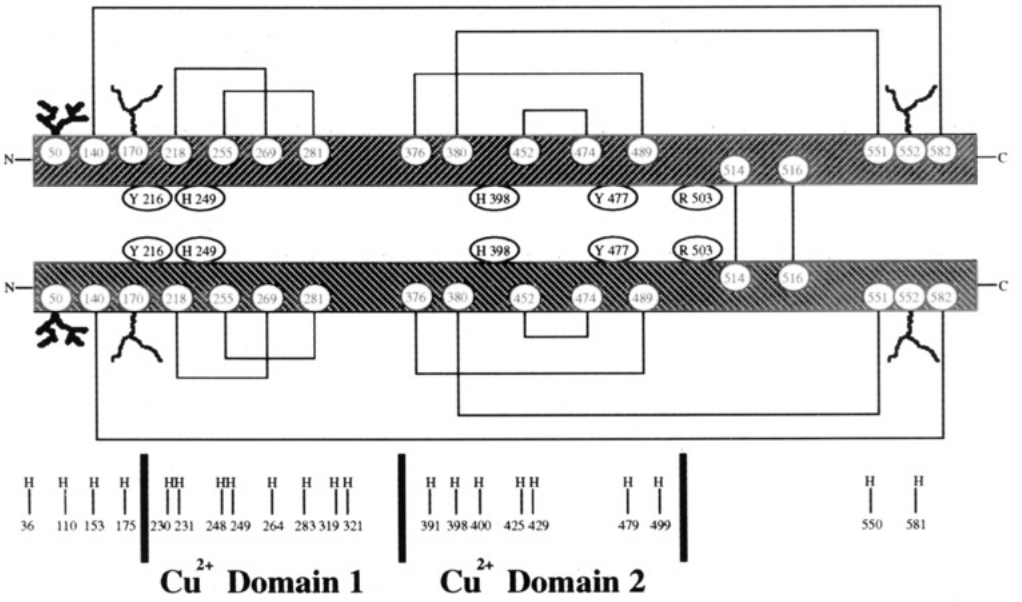

FIGURE 11: Proposed structural model of dopamine β-hydroxylase. The model illustrates the relative location of intrachain and interchain disulfide bonds in a disulfide-bonded dimer, where the N-terminus starts at Ser26 and the C-terminus ends at Gly603 (Robertson et al., 1991). The positions of N-linked biantennary oligosaccharides are shown at Asn170 and Asn 552, and the position of the high mannose oligosaccharide is shown at Asn50 (Robertson et al., 1991). The sites of mechanism-based inactivation by 6-hydroxybenzofuran (Tyr477) and phenylhydrazine (Tyr477, His249, and Arg503) (Farrington et al., 1990) are shown, and as well as the sites of inactivation by *p*-cresol (Tyr216) and β-ethynyltyramine (His398) (DeWolf et al., 1988, 1989). The relative positions of the 21 histidines in the protein and the potential Cu^{2+} binding domains are illustrated at the bottom.

Stimulatory exocytosis of secretory vesicles results in extracellular release of dopamine β-hydroxylase (Viveros et al., 1968; Weinshilboum et al., 1971). In addition, constitutive release of dopamine β-hydroxylase from PC 12 cells (McHugh et al., 1985) and bovine adrenal medullary cells in primary cultures (Corcoran & Kirshner, 1990) has also been observed. Thus, it appears that there is a mechanism for passage of the enzyme through the cell membrane apart from stimulatory exocytosis. However, both stimulatory and constitutive release of the enzyme must occur by a mechanism whereby the proposed solvent-exposed, carbohydrate-containing surface of the enzyme can gain access to its most favored solution conditions, whether these are complete solvation of the carbohydrate or a partial solvation in the presence of signal peptide.

The complexity of events leading to expression and localization of dopamine β-hydroxylase all involve structural questions about the organization of enzyme tetramers. Microheterogeneity in the N-termini (Robertson et al., 1990; Wang et al., 1990; Taylor et al., 1989), microheterogeneity in the signal peptide length (Taljanidisz et al., 1989; Wang et al., 1990; Lewis et al., 1990), microheterogeneity in glycosylation (Margolis et al., 1984), and the extent of bound phospholipid (Taylor & Fleming, 1989) may suggest many models where tetramers are composed of dissimilar post-translationally modified monomers. For instance, one tetramer might contain four subunits with signal peptides, or four without signal peptides, or mixtures of subunits with signal peptides. Evidence from the purified membrane bound form indicates that there must be mixtures because only 20% of the enzyme contains signal peptide. Furthermore, pH dependent dissociation of tetramers into disulfide linked dimers (Saxena et al., 1985) suggests that soluble dimers might associate with membrane bound dimers. Clearly, a structural model of the enzyme would provide insights into the biology of its function.

The disulfide linkages described here provide a first model for rationalizing some of the physical features of bovine dopamine β-hydroxylase. They also suggest the complexity of folding required for expression of active enzyme, and may now be used as a guide to mutagenesis. Site-directed mutagenesis of the intersubunit disulfides could test the requirement of dimerization for assembly of active enzyme. Similarly, mutagenesis of the N- to C-terminal disulfide might be a probe of correct glycosylation, targeting to the chromaffin granule, and topological requirements for membrane association. Mutagenesis of the internal disulfide domains might be a probe of metal binding requirements, and the suggestion that two clusters of histidines overlap the internal disulfide domains provides a starting point for histidine mutagenesis to locate the metal-binding sites. The proposed model ultimately must be tested by determination of the three-dimensional structure, but it should provide a useful starting ground for experimentalists interested in structure/function studies of dopamine β-hydroxylase.

ACKNOWLEDGMENT

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