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Bovine Dopamine β-Hydroxylase, Primary Structure Determined by cDNA Cloning and Amino Acid Sequencing†

Naiyin Wang,‡ Christopher Southan,§ Walter E. DeWolf, Jr.,∥ Tim N. C. Wells,§ Lawrence I. Kruse,⊥,# and Robin J. Leatherbarrow*,‡

Department of Chemistry, Imperial College of Science and Technology, Exhibition Road, London SW7 2AZ, U.K., Department of Cellular Pharmacology and Department of Medicinal Chemistry, Smith Kline & French Research Limited, The Frythe, Welwyn, Hertfordshire AL6 9AR, U.K., and Department of Medicinal Chemistry, Smith Kline & French Laboratories, King of Prussia, Pennsylvania 19406

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ABSTRACT: A cDNA clone encoding bovine dopamine β-hydroxylase (DBH) has been isolated from bovine adrenal glands. The clone hybridizes to two oligonucleotide probes, one based on a previously reported active site peptide [DeWolf, W. E., Jr., et al. (1988) *Biochemistry 27*, 9093–9101] and the other based on the human DBH sequence [Lamouroux, A., et al. (1987) *EMBO J. 6*, 3931–3937]. The clone contains a 1.9-kb open reading frame that codes for the soluble form of bovine DBH, with the exception of the first six amino acids. Direct confirmation of 93% of the cDNA-derived sequence was obtained from cleavage peptides by protein sequencing and mass spectrometry. Differences were found between these two sequences at only two positions. Of the four potential N-linked carbohydrate attachment sites, two, Asn-170 and Asn-552, were shown to be partially and fully glycosylated, respectively. Within the 69% of the protein sequence confirmed by mass spectrometry, no other covalent modifications were detected.

Dopamine β-hydroxylase (DBH,[1] EC 1.14.17.1) is a tetrameric, copper-dependent glycoprotein that catalyzes oxidation of dopamine to norepinephrine in the biosynthesis of neurotransmitter [for a review, see Stewart and Klinman (1988a)]. The physiological importance of DBH and the role of copper in the oxygen scission and substrate hydroxylation have prompted a number of mechanistic and structural studies. Kinetic studies based upon isotope effects (Miller & Klinman, 1985), inhibitor characterization (Kruse et al., 1986; Fitzpatrick & Villafranca, 1987; Goodhart et al., 1987), and rapid-quench experiments (Brenner et al., 1989) have made some progress toward defining the chemical steps that occur during catalysis. Recent magnetic and spectroscopic studies of the prosthetic copper atoms (Scott et al., 1988; McCracken et al., 1988; Blumberg et al., 1989; Brenner & Klinman, 1989) have begun to correlate catalytic steps with copper redox state and ligand environment. The mechanism-based inactivators β-ethynyltyramine and *p*-cresol have been shown to be attached to sites that may be part of the domains which bind the phenethylamine substrate (DeWolf et al., 1988, Southan et al., 1990) and the copper atom(s) (DeWolf et al., 1989). The availability of cDNA-derived protein sequences for human DBH has allowed the location of these putative active site peptides to corresponding positions in the human polypeptide chain (Lamouroux et al., 1987; Kobayashi et al., 1989).

As part of our continuing investigation into the relationships between the structure and catalytic function of the bovine enzyme, this paper reports the molecular cloning and sequencing of a 2.2-kb cDNA coding for the soluble form of bovine DBH and the 3′ untranslated region. In addition, we directly confirm the protein sequence from combined Edman sequencing and FAB-MS data for peptides representing 93% of the DBH primary structure. Four sites of N-glycosylation are predicted from the cDNA sequence, and glycopeptides that correspond to two of these have been identified. The FAB-MS data support other evidence that the DBH contains neither covalently attached phosphate nor pyrroloquinoline quinone (PQQ).

Experimental Procedures

Preparation of Tryptic Peptides. DBH was S-carboxymethylated and then digested with trypsin as described previously (DeWolf et al., 1988). The peptides were separated by RP-HPLC using a Vydac C_4 250 × 4.6 mm column (Separations Group). Solvent A was 0.1% aqueous TFA, and solvent B was 0.1% TFA in acetonitrile. The resultant chromatogram is shown in Figure 1. A total of 67 peak fractions were collected manually. Fractions 63 and 65 contained peptides with covalently incorporated [^{3}H]-*p*-cresol and have been characterized in a previous study (DeWolf et al., 1988). Some fractions containing multiple N-terminal sequences were rechromatographed to obtain purer peptides prior to resequencing. This rechromatography was carried out under the same conditions as given in Figure 1 but with a 2 × 30 mm RP-300 phenyl cartridge (Applied Biosystems, Cheshire, U.K.) and a flow rate of 0.3 mL/min.

Analysis of Peptides Precipitated during Tryptic Digestion. After acidification of the tryptic digest a fraction of insoluble material was recovered as a pellet after bench centrifugation. Subsequently, 100 μL of saturated urea was added, the solution was clarified by a further centrifugation, and the supernatant was analyzed by RP-HPLC. Peptides were separated as de-

† The nucleic acid sequence in this paper has been submitted to GenBank under Accession Number J02909.

* To whom correspondence should be addressed.

‡ Imperial College of Science and Technology.

§ Department of Cellular Pharmacology, Smith Kline & French Research Limited.

∥ Smith Kline & French Laboratories.

⊥ Department of Medicinal Chemistry, Smith Kline & French Research Limited.

Present address: Department of Chemistry, Sterling Drug Inc., Malvern, PA 19355.

[1] Abbreviations: DBH, dopamine β-hydroxylase; RP-HPLC, reverse-phase high-performance liquid chromatography; Con-A, concanavalin A; FAB-MS, fast atom bombardment mass spectrometry; PTH, phenylthiohydantoin; TFA, trifluoroacetic acid; CM, carboxymethylated; GuHCl, guanidine hydrochloride; PAM, peptide α-amidating enzyme; PQQ, pyrroloquinoline quinone.

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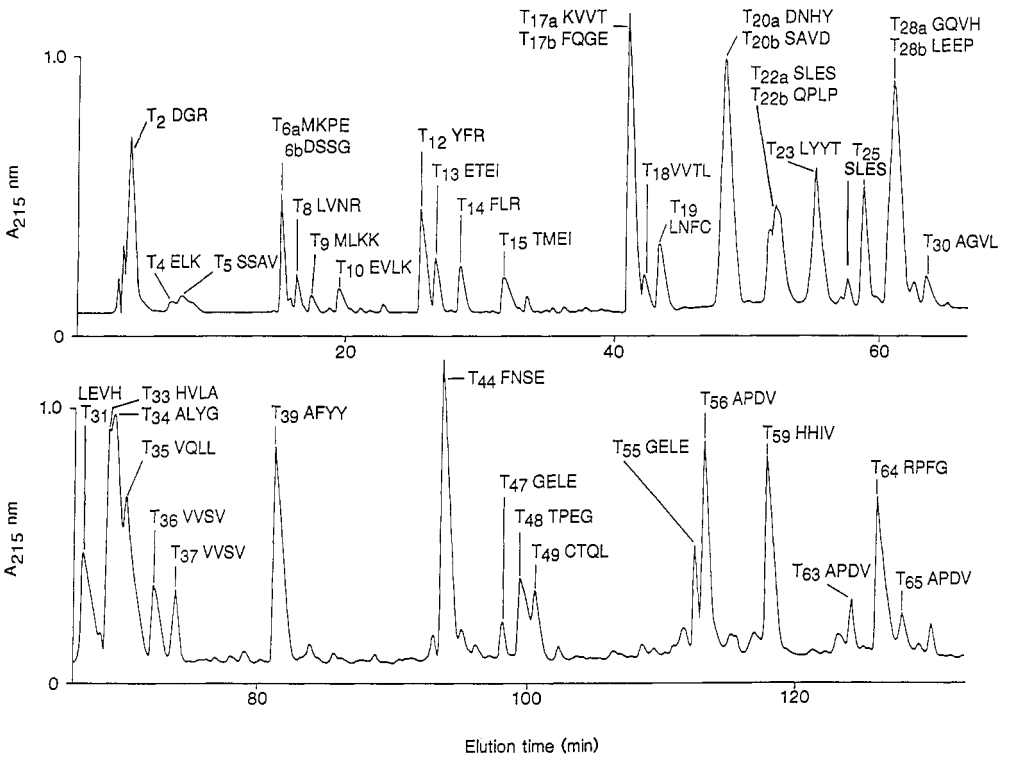


FIGURE 1: RP-HPLC separation of 20 μmol of tryptic peptides from *p*-cresol-inactivated and S-carboxymethylated bovine DBH. A Vydac C_4 (250 × 4.6 mm) column was used with a flow rate of 0.5 mL min^{-1}. Solvent A was 0.1% TFA; solvent B was 0.1% TFA in acetonitrile. Peptides were eluted with a gradient of 0–63% B in 126 min and collected manually as peak fractions. Identified peptides (Figure 4) are designated by their peak number and the sequence of the first four N-terminal residues (except for tripeptides). The suffixes a and b refer to multiple peptides identified in single fractions or after the rechromatography of that fraction.

scribed (see Figure 1 legend) except that a 2 × 50 mm Vydac phenyl column (Technicol, Macclesfield, U.K.) was used with a 0–70% gradient in 35 min at 0.3 mL/min.

Preparation of Overlapping Peptides. A total of 600 pmol of DBH was derivatized with 2-vinylpyridine to *S*-pyridylethylated cysteinyl residues (Friedman et al., 1970); 300 pmol of the modified DBH was cleaved with endoproteinase Glu-C (BCL, Sussex, U.K.). The same amount of native DBH in 100 μL of 70% TFA was cleaved with 0.5 mg of cyanogen bromide overnight at room temperature under nitrogen. After lyophilization 100 μL of 8 M GuHCl was added to improve peptide solubility. Both the Glu-C and CNBr cleavage mixtures were separated under the same conditions as described in Figure 1 except that a 2 × 250 mm Vydac C_4 column (Technicol, Cheshire, U.K.) was run at a flow rate of 0.2 mL/min.

Isolation of Glycopeptides. A small affinity column of concavalin A–Sepharose (Con-A, Pharmacia LKB, Milton Keynes, U.K.) was constructed in a 200-μL plastic pipet tip fitted into a 0.22-μm syringe filter (Gelman Sciences, Northampton, U.K.). The Con-A–Sepharose was packed into the tip (50-μL packed volume) and washed by a syringe with 2 mL of 5 mM potassium phosphate buffer, pH 7.0. A portion of the soluble fraction of the tryptic digest referred to above was reduced in volume by centrifugal evaporation (Gyrovap, V.A. Howe, U.K.) and redissolved in distilled water. After centrifugation at 8000 rpm for 10 min in a bench-top centrifuge (Beckman), a 100-μL aliquot of this, containing approximately 500 pmol of peptide mixture, was loaded onto the Con-A column and washed with 2 mL of phosphate buffer. Bound peptides were then eluted with 50 μL of phosphate buffer saturated with methyl α-D-mannoside. By a washing with a further 250 μL of phosphate buffer, an eluate of 300 μL was collected. This was analyzed by RP-HPLC using the same solvent conditions as described in Figure 1. A 1 × 100 mm Aquapore RP 300 C_8 microbore column was used with a 0–70% gradient in 35 min at 0.1 mL/min.

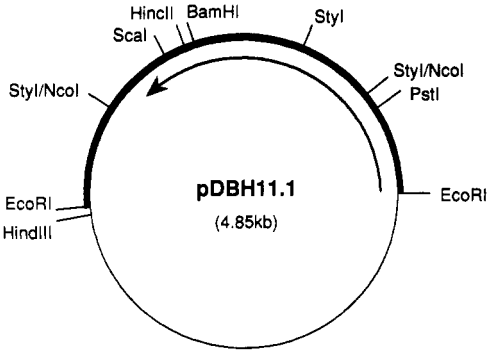


FIGURE 2: Restriction map of the pUC8 plasmid that contains the 2.2-kb *Eco*RI fragment from pDBH11.1 encoding the gene for the soluble form of DBH and the 3′ untranslated region.

Protein Sequencing. Depending on peak height of each HPLC fraction, aliquots of $^1/_{10}$ or $^1/_5$ were sequenced on an Applied Biosystems 477 pulsed-liquid phase instrument with a 120 on-line PTH analyzer. Polybrene was also loaded onto the sample disk but not precycled. Separation of the PTH derivatives was carried out under conditions specified by the manufacturer except for a lowering of the tetrahydofuran concentration in solvent A to 4% and a lowering of the pH to 3.8 with acetic acid. Under these conditions it was possible to identify Cys residues by the elution position of PTH-CM-Cys at 20 s prior to PTH-Ser.

Mass Spectral Analysis of Tryptic Peptides. Aliquots of RP-HPLC fractions containing between 2 and 5 nmol of tryptic peptides were concentrated to a final volume of 10 μL on a centrifugal evaporator. A 2-μL aliquot was loaded onto a target previously smeared with 2–4 μL of glycerol/thioglycerol (1:1), followed by a 1-μL aliquot of 0.1 M HCl. Fast

```
1     GAATTCCGCCCCTTCCCCTTCCACATCCCCCTGGACCCCGAGGGGACCCTGGAG    54
                P  F  P  F  H  I  P  L  D  P  E  G  T  L  E
55    CTGTCCTGGAACATCAGCTATGCGCAGGAGACCATCTACTTCCAGCTCCTGGTG   108
       L  S  W  N  I  S  Y  A  Q  E  T  I  Y  F  Q  L  L  V
109   CGGGAGCTCAAGGCTGGTGTCCTGTTTGGGATGTCGGACCGAGGGGAGCTGGAG   162
       R  E  L  K  A  G  V  L  F  G  M  S  D  R  G  E  L  E
163   AATGCTGACTTGGTGGTGCTCTGGACTGACAGGGACGGCGCCTACTTTGGGGAT   216
       N  A  D  L  V  V  L  W  T  D  R  D  G  A  Y  F  G  D
217   GCCTGGAGTGACCAGAAGGGGCAGGTCCACCTGGACTCCCAGCAGGATTACCAG   270
       A  W  S  D  Q  K  G  Q  V  H  L  D  S  Q  Q  D  Y  Q
271   CTTCTGCGGGCACAGAGGACTCCAGAAGGCCTGTACCTGCTCTTCAAGAGGCCT   324
       L  L  R  A  Q  R  T  P  E  G  L  Y  L  L  F  K  R  P
325   TTTGGCACCTGTGACCCCAACGACTACCTCATCGAGGACGGCACCGTCCACCTG   378
       F  G  T  C  D  P  N  D  Y  L  I  E  D  G  T  V  H  L
379   GTGTATGGATTCCTGGAGGAGCCGCTCCGGTCGCTGGAGTCCATCAACACATCC   432
       V  Y  G  F  L  E  E  P  L  R  S  L  E  S  I  N  T  S
433   GGCTTGCACACGGGGCTGCAGAGGGTGCAGCTGCTGAAGCCCAGCATCCCGAAG   486
       G  L  H  T  G  L  Q  R  V  Q  L  L  K  P  S  I  P  K
487   CCGGCCCTGCCCGCGGACACGCGCACCATGGAGATCCGCGCCCCCGACGTCCTC   540
       P  A  L  P  A  D  T  R  T  M  E  I  R  A  P  D  V  L
541   ATCCCCGGCCAGCAGACCACGTACTGGTGCTACGTGACCGAGCTCCCGGACGGC   594
       I  P  G  Q  Q  T  T  Y  W  C  Y  V  T  E  L  P  D  G
595   TTCCCCCGGCACCACATCGTCATGTACGAGCCCATCGTCACCGAGGGCAACGAG   648
       F  P  R  H  H  I  V  M  Y  E  P  I  V  T  E  G  N  E
649   GCGCTGGTGCACCACATGGAGGTCTTCCAGTGCGCCGCCGAGTTCGAGACCATC   702
       A  L  V  H  H  M  E  V  F  Q  C  A  A  E  F  E  T  I
703   CCCCACTTCAGCGGGCCCTGCGACTCCAAGATGAAGCCGCAGCGGCTCAACTTC   756
       P  H  F  S  G  P  C  D  S  K  M  K  P  Q  R  L  N  F
757   TGCCGTCACGTGCTGGCCGCCTGGGCCCTGGGCGCCAAGGCCTTTTACTACCCA   810
       C  R  H  V  L  A  A  W  A  L  G  A  K  A  F  Y  Y  P
811   GAGGAAGCAGGCCTGGCCTTCGGGGGGCCCGGCTCCTCCAGATTTCTCCGCCTG   864
       E  E  A  G  L  A  F  G  G  P  G  S  S  R  F  L  R  L
865   GAAGTTCACTACCACAACCCACTGGTGATAACAGGCCGGCGCGACTCCTCGGGC   918
       E  V  H  Y  H  N  P  L  V  I  T  G  R  R  D  S  S  G
919   ATCCGCCTGTACTACACGGCTCGGCTGCGGCGCTTCGACGCGGGCATCATGGAG   972
       I  R  L  Y  Y  T  A  R  L  R  R  F  D  A  G  I  M  E
973   CTGGGCCTGGCGTACACGCCCGTGATGGCCATCCCCCCGCAGGAGACGGCCTTC  1026
       L  G  L  A  Y  T  P  V  M  A  I  P  P  Q  E  T  A  F
1027  GTCCTCACCGGCTACTGCACGGACAAGTGCACCCAGCTGGCCCTGCCCGCCTCA  1080
       V  L  T  G  Y  C  T  D  K  C  T  Q  L  A  L  P  A  S
1081  GGGATTCACATCTTCGCCTCTCAGCTCCACACGCACCTGACCGGCCGGAAGGTG  1134
       G  I  H  I  F  A  S  Q  L  H  T  H  L  T  G  R  K  V
1135  GTCACAGTGCTGGCCAGGGACGGCCGGGAGACAGAGATCGTGAACAGGGACAAC  1188
       V  T  V  L  A  R  D  G  R  E  T  E  I  V  N  R  D  N
1189  CACTACAGCCCACACTTCCAGGAGATCCGCATGTTGAAGAAGGTCGTGTCTGTC  1242
       H  Y  S  P  H  F  Q  E  I  R  M  L  K  K  V  V  S  V
1253  CAGCCGGGAGACGTGCTCATCACCTCTTGCACATACAACACGGAAGACAGGAGG  1296
       Q  P  G  D  V  L  I  T  S  C  T  Y  N  T  E  D  R  R
1297  CTGGCCACCGTGGGGGGCTTCGGGATCCTCGAGGAGATGTGCGTCAACTATGTG  1350
       L  A  T  V  G  G  F  G  I  L  E  E  M  C  V  N  Y  V
1351  CACTACTACCCCCAGACGCAGCTGGAGCTCTGCAAGAGCGCCGTGGACCCTGGC  1404
       H  Y  Y  P  Q  T  Q  L  E  L  C  K  S  A  V  D  P  G
1405  TTCCTGCACAAGTACTTCCGCCTCGTGAACAGGTTCAACAGCGAGGAAGTCTGC  1458
       F  L  H  K  Y  F  R  L  V  N  R  F  N  S  E  E  V  C
1459  ACCTGCCCCCAGGCGTCTGTCCCTGAGCAGTTTGCCTCCGTGCCCTGGAACTCC  1512
       T  C  P  Q  A  S  V  P  E  Q  F  A  S  V  P  W  N  S
1513  TTCAACCGCGAGGTGCTCAAGGCCCTGTACGGCTTCGCACCCATCTCCATGCAC  1566
       F  N  R  E  V  L  K  A  L  Y  G  F  A  P  I  S  M  H
```

```
1567  TGCAACAGGTCCTCGGCCGTCCGCTTCTAGGGCGAGTGGAATCGGCAGCCCCTG  1620
        C  N  R  S  S  A  V  R  F  Z  G  E  W  N  R  Q  P  L
1621  CCTGAGATCGTGTCCAGGTTGGAAGAGCCCACCCCTCACTGCCCAGCCAGCCAG  1674
        P  E  I  V  S  R  L  E  E  P  T  P  H  C  P  A  S  Q
1675  GCTCAGAGCCCCGCCGGCCCCACCGTGCTGAACATCAGTGGGGGCAAAGGCTGA  1728
        A  Q  S  P  A  G  P  T  V  L  N  I  S  G  G  K  G  Z
1729  ACGTGGGCAGTCTCCTCGCTCCCCTCACCATGCTGTCCTGCGGGCTCACAGCAG  1782

1783  CCCTGTGCACCCCCTACTCTGTGAAGACCCCCATGGAATAGCCCAGCACGGAGG  1836

1837  GCTGGACCAAGCCACCACCTGAGACCAGGGTCCGGTCCAGCTTTCTCCCCCAGG  1890

1891  GACCCCCTGCATGGCTGAGAGGGTCCCGTGACAACTTTTGCTGACCCACCGAGG  1944

1945  CCCAGGTGGACCAGGACCCTTGCACACCCTTTGACACAGCATAAGAGCAACCCC  1998

1999  TTTTGGAAGTCTAGAGTCCAGAGCCCCGAGAGCCCTGCCATCTCGCTGGGGCTG  2052

2053  GGGGGTGCCCGTCTCCTGGGACACGAGCCACGACCGGACGCGGGCCAGACTCCC  2106

2107  GGAGCTGTCCCGGCCCCGCGTCCCCAGCCCCCACGGAGGGTGATCGGTGTTGAG  2160

2161  TGTGACGGGTGCAAGTGCTGTTGTACTTAAATGTGTCCCTGCAGAAAAAAAAAA  2214

2215  AAAAAAAAAAAAAAAAA                                        2232
```

FIGURE 3: Nucleotide sequence of bovine DBH cDNA from clone DBH 11.1. The upper-case sequence is the complete reading frame for the protein including the stop codons (Z), but with the exception of the first six amino acids and the signal sequence.

atom bombardment analyses were carried out on a VG Analytical ZAB-2SE or VG 70-250-SEG high-field mass spectrometer (Manchester, U.K.) operating at an accelerating voltage of 8 kV. A cesium ion gun was used to generate ions for the mass spectra, which were recorded on a VAX PDP 11-250J data system. Mass calibration was performed with cesium iodide or cesium iodide/glycerol (1:1).

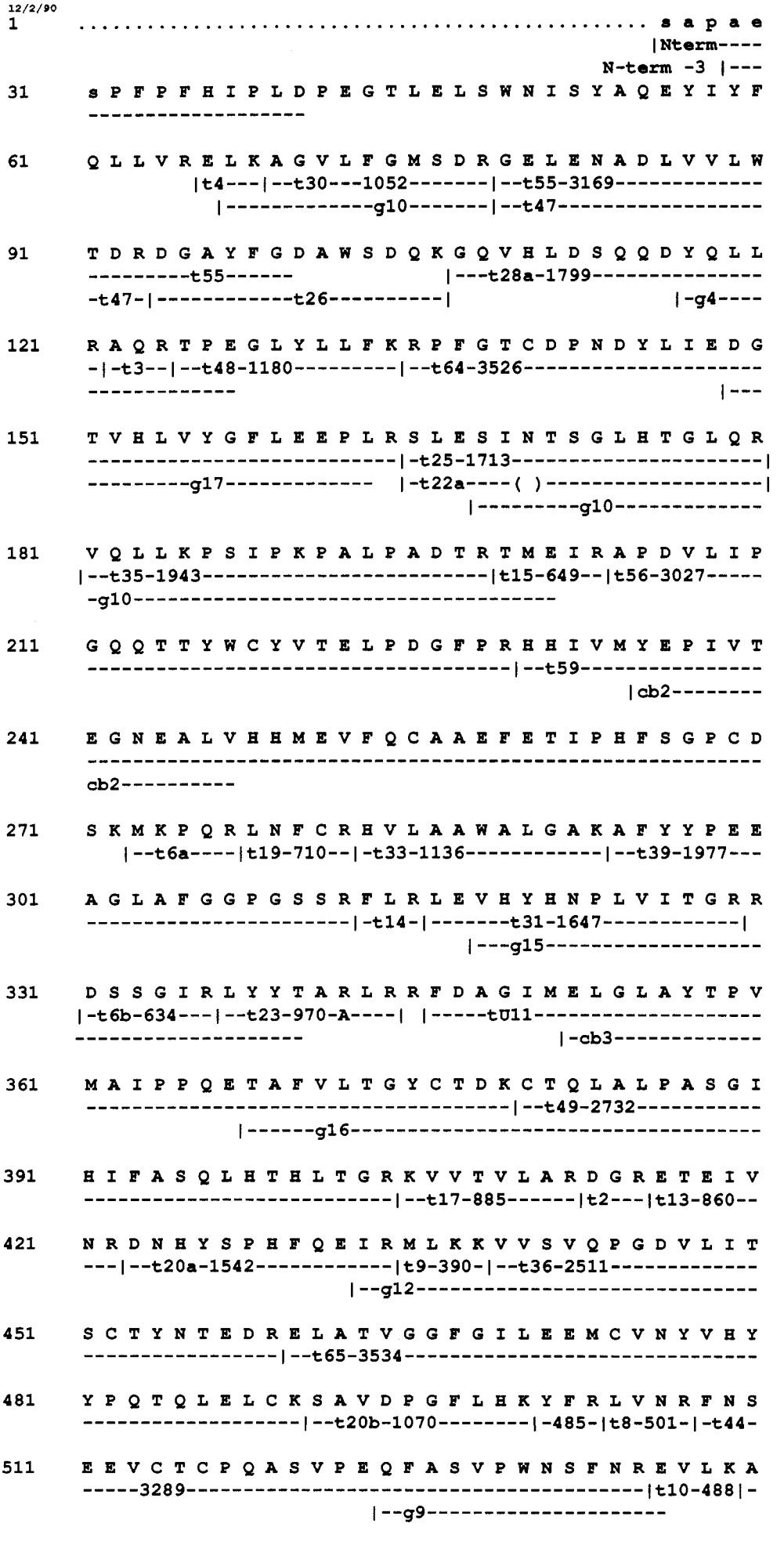
Design and Preparation of Oligonucleotide Probes. Two oligonucleotide probes were designed to facilitate the cloning of the gene. The first was based on the active site peptide sequence obtained from the bovine enzyme (DeWolf et al., 1988). It spans the protein sequence between Gln-212 and Val-220 and has the sequence 5′-GTC GTC TGG TGC ATG ACC ACG ATG CA-3′. The second probe was based on the sequence located in the C-terminal region of the human gene (Lamouroux et al., 1987) in an area rich in the amino acids that are low in codon redundancy. It spans the protein sequence from Glu-471 to Pro-482 and has the sequence 5′-CTC CTC TAC ACA CAG TTG ATG CAC GTG ATG GG-3′. The nucleotide sequence of both of these probes was optimized for bovine codon preferences. The bovine codon utilization table was constructed from the database within the Microgenie program (Queen & Korn, 1984).

A third probe was designed on the basis of the sequence of the active site peptide obtained in the covalent modification experiments using *p*-cresol (DeWolf et al., 1988). In this case deoxyinosine was used for the unknown third bases in each codon (Ohtsuka et al., 1985). This approach was expected to increase the efficiency of the probe, since deoxyinosine should not seriously destabilize DNA duplex formation. However, the deoxyinosine-containing probe failed to identify the positive clones. It is assumed that the large number of deoxyinosines in this probe contributed to a large amount of nonspecific binding. The oligonucleotide was based on the protein sequence between Ile-209 and Glu-222 and had the sequence 5′-TAI GGI CCI GTI GTI TGI TGI ATI ACC ACI ATI CAI TGI CT-3′.

All the oligonucleotides were synthesized on an Applied Biosystems 380B synthesizer. They were purified by electrophoresis through 20% polyacylamide gels containing 7 M urea. Gel bands were identified by UV shadowing at 254 nm, excised, and extensively dialyzed against distilled water. Probes were then 5′-end labeled with [γ-^{32}P]ATP (Amersham) and T4 polynucleotide kinase (NBL).

Screening of cDNA Library. The cDNA library, derived from bovine adrenal medulla cDNA, was cloned into λgt11 with *Eco*RI linkers. Recombinant plaques (2×10^4) were grown in *Escherichia coli* Y1088 (American Tissue Culture Collection No. 37195) and screened by hybridization to the ^{32}P-labeled oligonucleotide. Plaques were lifted onto Hybond membranes (Amersham, U.K.) and prehybridized at 56 °C for 1 h in a solution containing 5× Denhardt's buffer, 5× SSPE (0.9 M NaCl, 0.05 M NaH_2PO_4/Na_2HPO_4, pH 7.7, 5 mM EDTA), and 0.2% sodium dodecyl sulfate. Hybridization was carried out in the same buffer, with labeled probe added to give 1×10^6 cpm/mL. Filters were washed at 25 °C in 6× SSC (0.9 M NaCl, 90 mM sodium citrate, pH 7.0) for 1 h and then at 56 °C for 10 min. Southern blots of the cDNA library were carried out according to Manniatis et al. (1982).

DNA Sequencing Strategy. After digestion with *Eco*RI the insert from DBH11 was subcloned into both M13mp18 and pUC118. DNA sequence analysis was carried out by the Sanger dideoxy chain termination method (Sanger et al., 1977) using deoxyadenosine α-[^{35}S]thiotriphosphate (Amersham) and Sequenase (U.S. Biochemicals). A set of overlapping deletions was obtained with DNase I in the presence of Mn^{2+} as described by Lin et al. (1985). The method was modified in that Geneclean (Stratagene) was used instead of ethanol precipitation for purification of DNA.


12/2/90
1 ..... s a p a e
|Nterm----
N-term -3 |---
31 s P F P F H I P L D P E G T L E L S W N I S Y A Q E Y I Y F
61 Q L L V R E L K A G V L F G M S D R G E L E N A D L V V L W
|t4---|--t30---1052-------|--t55-3169----------
|----------g10-------|--t47----------
91 T D R D G A Y F G D A W S D Q K G Q V H L D S Q Q D Y Q L L
---------t55------ |---t28a-1799----------
-t47-|-----------t26----------| |-g4----
121 R A Q R T P E G L Y L L F K R P F G T C D P N D Y L I E D G
-|-t3--|--t48-1180---------|--t64-3526----------
---------- |---
151 T V H L V Y G F L E E P L R S L E S I N T S G L H T G L Q R
-------------------------|-t25-1713----------|
---------g17---------- |-t22a----( )----------|
|---------g10----------
181 V Q L L K P S I P K P A L P A D T R T M E I R A P D V L I P
|--t35-1943----------|t15-649--|t56-3027-----
-g10----------
211 G Q Q T T Y W C Y V T E L P D G F P R H H I V M Y E P I V T
----------|--t59----------
|cb2--------
241 E G N E A L V H H M E V F Q C A A E F E T I P H F S G P C D
----
cb2----------
271 S K M K P Q R L N F C R H V L A A W A L G A K A F Y Y P E E
|--t6a----|t19-710--|-t33-1136----------|--t39-1977---
301 A G L A F G G P G S S R F L R L E V H Y H N P L V I T G R R
----------|-t14-|-------t31-1647----------|
|---g15----------
331 D S S G I R L Y Y T A R L R R F D A G I M E L G L A Y T P V
|-t6b-634---|--t23-970-A----| |-----tU11----------
---------- |-cb3----------
361 M A I P P Q E T A F V L T G Y C T D K C T Q L A L P A S G I
----------|--t49-2732----------
|------g16----------
391 H I F A S Q L H T H L T G R K V V T V L A R D G R E T E I V
----------|--t17-885------|t2---|t13-860--
421 N R D N H Y S P H F Q E I R M L K K V V S V Q P G D V L I T
---|--t20a-1542----------|t9-390-|--t36-2511----------
|--g12----------
451 S C T Y N T E D R E L A T V G G F G I L E E M C V N Y V H Y
----------|--t65-3534----------
481 Y P Q T Q L E L C K S A V D P G F L H K Y F R L V N R F N S
----------|--t20b-1070--------|-485-|t8-501-|-t44-
511 E E V C T C P Q A S V P E Q F A S V P W N S F N R E V L K A
-----3289----------|t10-488|-
|--g9----------

```
541   L Y G F A P I S M H C N R S S A V R F Z G E W N R Q P L P E
      -------t34----------( )-|-t5-519--|--Q-t17b-935-|t22b-----
                                                     |--g3----------
571   I V S R L E E P T P H C P A S Q A Q S P A G P T V L N I S G
      --1038-|--t28b--------------------

601   G K G
```

FIGURE 4: Composite primary structure map of bovine DBH. The residue numbering corresponds to that used for human DBH (Lamouroux et al., 1987). The upper-case sequence given was translated from the cDNA. Dashed lines underneath indicate residues that were confirmed by protein sequencing. The N-terminal analysis gave two sequences frame shifted by three residues. The first six residues, given in lower case, were not available in the cDNA. The numbers adjacent to peptide designations refer to the FAB-MS detection of the positively charged molecular ions ($M + H^+$) independently confirming the peptide structures. A number of overlapping peptides are also indicated as dashed lines. Those labeled "g" are from the endoproteinase Glu-C digest, and those labeled "cb" are from CNBr cleavage. Two gaps, indicated in brackets, correspond to identified N-glycosylation sites where no PTH was detected. The two differences between the cDNA and protein sequence, as discussed under Results, are shown beneath the cDNA at positions 342 and 560.

Sequence Data Analysis. This was performed on a VAX/VMS system using the suite of programs purchased from the University of Wisconsin Genetics Computer Group (UWGCG) (Devereux et al., 1984). Sequence database searches were carried out with the FASTA program, searching the current Dayhoff library of 20 535 protein sequence entries. Human DBH and other copper binding proteins were found in the current SwissProt protein database (release 10.03.89). Other protein sequences tested for comparison were obtained by use of TRANSLATE on the appropriate cDNA sequences retrieved from GenBank. References for the sequences tested are included in the database entries. Dot-plot comparisons were done with COMPARE; alignments were tested with both the GAP and BESTFIT programs.

Results

Molecular Cloning and Sequence of 2.2-kb Fragment. A cDNA library was constructed from bovine adrenal medulla, a tissue that expresses DBH at high activity. A total of 2×10^5 clones were screened with two oligonucleotides, A (26-mer) and B (35-mer). Seven positive clones were identified with oligonucleotide A, of which three remained positive after repeated plaque purification. These three clones also hybridized to oligonucleotide B and were named DBH6, DBH9, and DBH11. Clone DBH11 contained the largest insert and so was used to obtain DNA sequence. The insert in DBH11 was found to contain two internal *Eco*RI sites and so produced three *Eco*RI digestion products with sizes of 2.2, 1.0, and 0.5 kb. These three *Eco*RI fragments were each cloned into pUC118, giving pDBH11.1, pDBH11.2, and pDBH11.3, respectively. The restriction map of pDBH11.1 is shown in Figure 2. The complete nucleotide sequence of DBH11.1 is given in Figure 3. This fragment contained the cDNA for DBH except for the region coding for the first six amino acids and the signal sequence. The other two *Eco*RI fragments, from pDBH11.2 and pDBH11.3, were also sequenced. No regions in these two frgments showed significant homology with the published human DBH signal sequence (Lamouroux et al., 1987). As a consequence of the high degree of sequence similarity between human and bovine DBH, we have adopted the convention of using same residue numbering as that used for the human DBH (Lamouroux et al., 1987).

Analysis of Peptides. The RP-HPLC separation of the soluble fraction of tryptic peptides is shown in Figure 1. Alignments for these peptides with the cDNA-derived sequence are shown in Figure 4. Overlapping peptides from the endoproteinase Glu-C digest and the CNBr cleavage give additional confirmation of the continuity of the peptide chain. Analysis of tryptic RP-HPLC fractions by FAB-MS provided the correct mass weight ($M + H^+$) for all peptides that gave clear molecular ion signals within the mass range scanned.

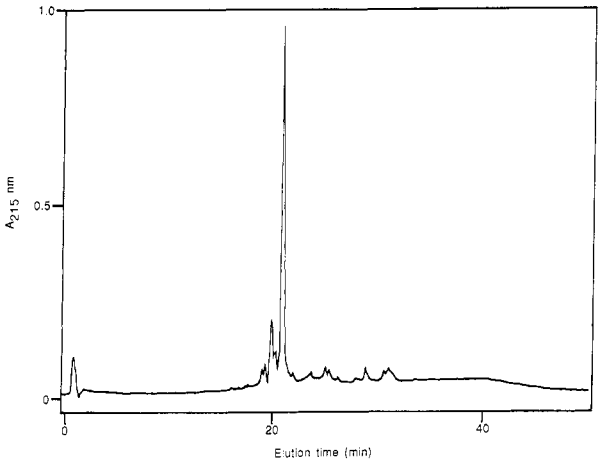


FIGURE 5: Analysis of tryptic glycopeptides eluted from a Con-A column. Solvent A was 0.1% TFA/water; solvent B, 0.1% TFA/acetonitrile. A gradient of 0–70% B in 35 min was run at a flow rate of 100 μL/min on a 1 × 100 mm Aquapore RP300 C_8 column (Applied Biosystems). 50 μL of the Con-A eluate of 300 μL was injected (80 pmol). The peak was collected for protein sequence analysis.

From the accumulated protein sequence and FAB-MS data we confirmed 93% of the primary structure predicted from the cDNA for the mature protein (i.e., excluding the signal peptide). Sections of cDNA sequence not supported by peptide data are limited to positions 41–65, 271–272, 345, 552, and 589–603 (Figure 4).

Within the confirmed sections there are only two discrepancies between the cDNA and protein sequence data. Giving the cDNA translation first, these are 342 Arg/Ala and 560 Z(stop codon)/Gln. The polypeptide chain clearly proceeded past this point, and the assignment of Gln at position 560 was confirmed by FAB-MS of peptide T17b. In addition, the presence of a stop codon at this position would lead to a protein much smaller than the observed molecular size of the unglycosylated protein (Speedie et al., 1985).

Posttranslational Modifications. DBH is known to be a glycoprotein capable of binding to Con-A columns. Structural analysis of the tryptic peptides led to the identification of two N-linked glycosylation sites of the Asn-X-Ser type. During the sequencing of peptide T34 (residues 540–553 in Figure 4) no PTH derivative could be identified at position 552 where Asn was predicted from the cDNA. When the same peptide fraction was analyzed by FAB-MS, no strong molecular ion signals could be detected within the mass range scanned. When the tryptic digest of DBH was applied to a Con-A column, only one peptide was eluted (Figure 5). Subsequent sequence analysis showed that this peptide was identical with T34. These observations are consistant with glycosylation of Asn-552.

Two chromatographically distinct forms of tryptic peptide, T22 and T25, gave identical sequences spanning residues 165–180, exept that in T22 no PTH was identified at position 170 corresponding to PTH-Asn in T25. Analysis by FAB-MS was able to confirm the appropriate peptide mass weight for T25 but did not give a clear molecular ion signal for T22. These observations can be explained by partial glycosylation of Asn-170 resulting in T22 eluting earlier in RP-HPLC than its nonglycosylated counterpart, T25. The inability of T22 to bind to the Con-A column successfully used for the isolation of T34 as described implies that the sugar chains attached to Asn-170 and -552 are different in their affinity for Con-A. The former may therefore be of the complex tri- or tetraantennary type (Osawa & Tsuji, 1987).

Computer Analysis of Sequence Data. When a dot-matrix plot was used to compare the bovine DBH sequence against itself, diagonal patterns indicative of internaly repeated ancestral domains were not detected (Collins & Coulson, 1987). The same type of comparison between human and bovine DBH at high filtration showed increased sequence conservation in the central section of the molecule encompassing residues 230–440. This observation was supported by testing sectional alignments with the GAP program. The similarity scores when tested by sections were 85% for residues 25–230, 95% for 230–340, and 93% for 440–603. A search of the current Dayhoff protein sequence library with the bovine DBH sequence displayed the expected matches with the human sequence. The only other protein showing a significant similarity score over an extended section of sequence was the peptide C-terminal α-amidating enzyme (PAM) from *Xenopus laevis*. The significance of sequence similarity between these type II copper mixed-function oxidases has already been described in detail (Southan & Kruse, 1989).

Structural relationships between DBH and other proteins have been proposed, specifically with the β-adrenergic receptor (Shorr et al., 1987) and with tyrosine hydroxylase (TH) and phenylethanolamine *N*-methyl transferase (Joh et al., 1983). Using three sequence comparison programs, each utilizing different comparison algorithms, we were unable to detect significant similarity between DBH and any of these proteins. The immunological cross-reactivity observed by Shorr et al. (1987) could be explained by incomplete removal of common carbohydrate components by Endo-F (Tarentino et al., 1985). The comparative peptide mapping and nucleic acid hybridization data for DBH and TH presented by Joh et al. (1983) are difficult to reconcile with the absence of amino acid sequence similarity.

Discussion

This paper describes the isolation and characterization of a 2.2-kb DNA clone encoding the soluble form of DBH and its 3′ untranslated region. In addition, supporting protein sequence information was obtained from 52 cleavage peptides, 2 of which were identified as glycopeptides.

It is unusual for cDNA sequences of this size to be supported by extensive protein sequence information. However, the importance of using peptide structural data to identify cloning errors (Anderegg et al., 1988) and posttranslational modifications (Greer et al., 1988) has become increasingly recognized. The combined approach used in this work has the advantages of revealing any discrepancies between the two methods of determining protein sequence and allowed us to identify some posttranslation modifications.

Two differences were found between the cDNA translation and the peptide sequence. For the 342 Arg/Ser alternatives a change in two of the bases is required, converting the codon

```
  1 ------------------------SAPAESPFPFHIPLDPEGTLELSWN
    MREAAFMYSTAVAIFLVILVAALQG...R...L.Y........S......

 51 ISYAQETIYFQLLVRELKAGVLFGMSDRGELENADLVVLWTDRDGAYFGD
    V.YT..A.H......R.........................G.T...A.

101 AWSDQKGQVHLDSQQDYQLLRAQRTPEGLYLLFKRPFGTCDPNDYLIEDG
    ........I...P.......QV.......T............K.......

151 TVHLVYGFLEEPLRSLESINTSGLHTGLQRVQLLKPSIPKPALPADTRTM
    .................A..G...QM..........N..E.E..S.AC..

201 EIRAPDVLIPGQQTTYWCYVTELPDGFPRHHIVMYEPIVTEGNEALVHHM
    .VQ..NIQ..S.E......IK...K..S....IK......K.........

251 EVFQCAAEFETIPHFSGPCDSKMKPQRLNFCRHVLAAWALGAKAFYYPEE
    ......P.MDSV.............D...Y.....................

301 AGLAFGGPGSSRFLRLEVHYHNPLVITGRRDSSGIRLYYTARLRRFDAGI
    ............Y.............E..N...........K....N...

351 MELGLAYTPVMAIPPQETAFVLTGYCTDKCTQLALPASGIHIFASQLHTH
    .....V.........R....I..............P.............

401 LTGRKVVTVLARDGRETEIVNRDNHYSPHFQEIRMLKKVVSVQPGDVLIT
    ..........V.....W....Q.....................H.......

451 SCTYNTEDRELATVGGFGILEEMCVNYVHYYPQTQLELCKSAVDPGFLHK
    ........................................T...A...Q.

501 YFRLVNRFNSEEVCTCPQASVPEQFASVPWNSFNREVLKALYGFAPISMH
    ..H.I....N.D.........SQ..T.........D......S.......

551 CNRSSAVRFQGEWNRQPLPEIVSRLEEPTPHCPASQAQSPAGPTVLNISG
    ..K..........L....KVI.T......Q..T..GR.......VS.G.

601 GKG
    ...
```

FIGURE 6: Alignment of bovine DBH protein sequence (top) with the human sequence (Lamouroux et al., 1987). Dots below the human sequence mark identical residues.

CGG into AAG. The other alteration, 560 stop/Gln, requires a single base change, TAG/CAG. One possible explanation for these differences is that they represent population polymorphisms between the bovine samples from which the cDNA library was constructed and that from which the protein was purified. An alternative possibility is that the clone contains low-frequency transcriptional errors introduced by the AMV reverse transcriptase originally used to produce the cDNA library (Takeuchi et al., 1988).

Fifty-eight residues of protein sequence have previously been reported for bovine DBH (McCafferty & Angeletti, 1987). Our confirmed sequence data agree with their assignments except for Lys-106 and Ala-362 where they have reported Arg and Glu, respectively. The existence of polymorphisms and/or sequencing discrepancies is further illustrated by comparison of the two versions of human DBH. This reveals two differences at positions 197 Thr/Ala and 534 Cys/Arg between the human cDNA sequences from Lamouroux et al. (1987) and Kobayashi et al. (1989). These amino acid changes require a single-base and a two-base difference, respectively. The corresponding bovine amino acids at these positions are Thr-197 and Arg-534. In addition, Kobayashi et al. (1989) have detected a possible polymorphism, Ala/Ser-304, between the cDNA and genomic clones.

Comparison of human and bovine DBH shows that each sequence contains four potential Asn-X-Ser/Thr glycosylation sites (Figure 6). However, only three of these positions, Asn-50, Asn-170, and Asn-552, are conserved between the two species. For bovine DBH we have provided evidence for complete glycosylation at Asn-552 and partial glycosylation of Asn-170. This is consistent with a compositional heterogeneity averaging six oligosaccharide chains per tetramer

reported by Margolis et al. (1984).

In addition to glycosylation, other types of posttranslational modifications have been suggested for DBH. The synthesis of dopamine in the brain is regulated by a specific protein kinase that is known to phosphorylate the first enzyme on the pathway, tyrosine hydroxylase (Yamauchi et al., 1981). In the sequence of human DBH, Lamouroux et al. (1987) identified a potential site of regulatory phosphorylation at Ser-322. The bovine sequence contains the same potential site within the sequence Arg-Arg-Asp-Ser-Ser (positions 329–332) although the presence of Arg-330 in the bovine protein (instead of Asn in human DBH) would be expected to shift the specificity of phosphorylation from calmodulin- to cAMP-dependent kinases (Pinna et al., 1986). However, our FAB-MS molecular ion determination for T6 (Figure 4) excludes the presence of phosphate on Ser-332 or Ser-333. This result, in combination with data from other investigations (Stewart & Klinman, 1988b; Sabban et al., 1983; McHugh et al., 1985), implies that DBH activity is not regulated by phosphorylation/dephosphorylation. Van der Meer et al. (1988) have reported the stoichiometric covalent incorporation of pyrroloquinoline quinone (PQQ), putatively bound to lysine, as a cofactor in bovine DBH, although another investigation failed to confirm this finding (Robertson et al., 1989). Our extensive peptide characterization failed to detect any covalently bound PQQ.

We have confirmed the presence of a "ragged" N-terminal sequence in soluble preparations of native DBH containing two sequences of approximately equal amounts out of phase by three residues (Figure 4). This agrees with previous reports (Skotland et al., 1977; Joh & Hwang, 1986; Taylor et al., 1989). The consistent observation of this N-terminal heterogeneity suggests that it is not a purification artifact. This type of posttranslational modification has been detected in an increasing number of proteins and may arise as a consequence of multiple sites for the action of the signal peptidase or subsequent attack by an aminopeptidase (Jörnvall, 1987).

Comparison of the human and bovine DBH sequences indicates a higher degree of sequence conservation in the central section of the polypeptide chain (Figure 6). The most obvious constraints for evolutionary conservation in this enzyme would be for sections of primary structure involved in copper coordination, substrate binding, and/or catalysis. Recent studies on the bovine enzyme have shown that the catalytically competent form contains eight copper atoms per tetramer (Brenner & Klinman, 1989). Attempts to locate the copper binding site are based on three experimental observations. First, spectroscopic studies imply the involvement of histidine ligands in copper binding (Scott et al., 1989; Blumberg et al., 1989). Second, in superoxide dismutase, the only type II copper protein for which a three-dimensional structure is available (Tainer et al., 1982), the conserved copper binding motif is His-X-His. A search for this motif within the DBH sequence reveals His-Tyr-His at position 319 and His-Thr-His at position 398. These motifs are conserved between the human and bovine sequences and are therefore candidates for copper binding. Third, the proximity of residue 398 to the catalytic copper has been implicated previously from studies with the mechanism-based inactivator β-ethynyltyramine (DeWolf et al., 1989). Other residues implicated to be in the vicinity of the active site are those shown to be attachment sites for another mechanism-based inactivator, *p*-cresol. These positions, Tyr-216 (DeWolf et al., 1988) and Tyr-357 (Southan et al., 1990), are both conserved between the bovine and human sequences.

The degree of sequence conservation, the presence of copper coordination candidates, and the location of putative active site peptides all point toward the existence of a catalytic domain located in the central section of the polypeptide. Additional support for this idea comes from the recent description of sequence similarity between human DBH and bovine PAM (Southan & Kruse, 1989). The region of similarity extends between residues 190–485 of DBH. The homologous section in PAM, residues 50–320, has been independently implicated as a catalytic domain and includes a His-Thr-His motif in a position analogous to that conserved at position 398 in DBH.

Added in Proof

Since the submission of this paper a cDNA sequence for bovine DBH has been independently reported by Taljanidisz et al. (1989). Comparison of that sequence with the one determined in this work reveals some differences within the coding region. Listing the translations from this work first, the amino acid differences, followed by codon differences, are as follows: 198 Arg/Cys (CGC/TGC), 253 Phe/Phe (TTC/TTT), 260 Glu/Arg (GAG/CGA), 261 Thr/Asp (ACC/GAC), 262 Ile/His (ATC/CAT), 342 Arg/Ala (CGG/CGC), 560 stop/Gln (TAG/CAG), and 581 His/Gln (CAC/TAG). A further 10 differences are found in the 3′ noncoding region. Within the coding section the third-base codon change at 253 is "silent". The protein data from this work support our assignments at positions 198, 260–262, and 581 and the cDNA of Taljanidisz et al. (1989) for 342 and 560. The presence of these discrepancies between two independently determined cDNAs emphasizes the importance of confirmatory protein sequence data. We have recently obtained sequence data from the clone pDBH9 in which the codon at position 560 is GAG (Gln) rather than TAG (stop). This confirms the protein sequence data and suggests that clone pDBH11 contains a single base transcription error.

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