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Structure of Bovine Adrenal Dopamine β-Monooxygenase, As Deduced from cDNA and Protein Sequencing: Evidence That the Membrane-Bound Form of the Enzyme Is Anchored by an Uncleaved Signal Peptide[†,‡]

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ABSTRACT: A full-length cDNA for dopamine β-monooxygenase (DβM) from bovine adrenal glands has been cloned and sequenced. The soluble and membrane-derived forms of DβM have also been sequenced from their N-termini. While the observed sequences for the soluble protein correspond to those previously reported [Joh, T. H., & Hwang, O. (1986) *Ann. N.Y. Acad. Sci. 493*, 343-350], the heavy subunit of membrane-derived enzyme is found to contain a unique N-terminus. Alignment of this N-terminus with that deduced from cDNA cloning indicates identity at 22 (and possibly 26) out of 27 residues. This comparison leads us to conclude that the membranous form of bovine DβM retains an uncleaved N-terminal signal peptide as the source of membrane anchoring.

Dopamine β-monooxygenase (DβM, EC 1.14.17.1) catalyzes the conversion of dopamine to the neurotransmitter and hormone norepinephrine within the catecholamine-secreting vesicles (chromaffin granules) of the adrenal medulla. The presence of DβM as both a soluble and membrane-bound protein is well documented, with an approximately equal distribution of enzyme activity between these forms (Winkler & Carmichael, 1982). DβM is not unique in this regard, since two additional vesicular proteins—enkephalin convertase (Fricker et al., 1986) and peptidylglycine α-amidating monooxygenase (Diliberto et al., 1987)—have also been demonstrated to exist in dual forms. A major unresolved question in the area of DβM catalysis has been the structural difference and precursor-product relationship between the soluble and membranous forms of the enzyme.

Early investigations of DβM from bovine adrenal glands had suggested that both enzyme forms were comprised of four, identically glycosylated subunits with a molecular size of ca. 75 kDa (Wallace et al., 1973; Foldes et al., 1972; Hortnagel et al., 1972; Craine et al., 1973). More extensive investigations revealed *two* major protein bands at 72 and 75 kDa, with the membranous enzyme showing a higher proportion of the 75-kDa band (Saxena & Fleming, 1983; Speedie et al., 1985).

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Both Sabban et al. (1983) and McHugh et al. (1985) studied the biosynthesis of rat DβM in PC12 pheochromocytoma cells, observing a posttranslational processing of an initially formed 77-kDa membranous subunit to a soluble 73-kDa subunit with a final stoichiometry of ca. 1:1. This apparent precursor–product relationship was preserved in the presence of tunicamycin (between a 67-kDa, membranous subunit and a soluble, 63-kDa subunit), suggesting, first, that the difference between soluble and membranous enzyme is not due to differences in glycosylation and, second, that the source of membrane attachment lies with the heavier subunit (Sabban et al., 1983).

In light of the above results, a simple model had been advanced, invoking a hydrophobic peptide at the C- or N-terminus of membranous DβM as the source of membrane anchoring [for a recent review see Stewart and Klinman (1988a)]. This view was supported by the observations of Bjerrum et al. (1979), who found evidence for immunochemically identical forms of hydrophilic and amphiphilic forms of DβM, and of Slater et al. (1981) and Saxena and Fleming (1983), who reported that membranous DβM contains a slightly different peptide map than soluble enzyme. Membranous DβM has also been found to contain a higher proportion of hydrophobic amino acids (Slater et al., 1981) and to become functionally incorporated into liposomal membranes (Kent & Fleming, 1987).

The recent cloning of DβM from a human pheochromocytoma introduced a potential resolution of this long-standing structural problem (Lamouroux et al., 1987). However, examination of the sequence of the full-length human cDNA failed to reveal any hydrophobic domains—with the exception of a hydrophobic signal peptide at the N-terminus. Since S1 nuclease protection experiments implicated a single mRNA sequence for the coding region of both soluble and membrane-bound human DβM, Lamouroux et al. (1987) proposed posttranslational processing as the source of membrane attachment. In a subsequent study, Stewart and Klinman (1988b) failed to detect any evidence for a phosphatidylinositol glycolipid anchor in bovine adrenal DβM. This result was consistent with previous findings that DβM does not incorporate radiolabeled fatty acids upon maturation (McHugh et al., 1985) and appeared to rule our known posttranslational modification pathways in the generation of membranous DβM.

The above-described sequence of experimental findings refocused attention on a hydrophobic tail, i.e., the uncleaved, N-terminal signal peptide, as the membrane anchor in DβM (Stewart & Klinman, 1988b). However, when Fleming and co-workers performed N-terminal sequencing of DβM, almost identical sequences (lacking the signal peptide) were observed for both the soluble and membrane-derived forms of enzyme (Taylor et al., 1989). In the present study we have undertaken a multifaceted approach to the resolution of the structural difference between soluble and membranous DβM. We report the first successful cloning of a full-length cDNA for DβM from bovine adrenal glands. *Importantly, comparison of the sequence of this cDNA to protein sequences indicates retention of an N-terminal signal peptide in membranous DβM which is lacking in the soluble form of enzyme.* The failure of former workers to detect this structural difference may reflect contamination of membranous DβM preparations by a chromaffin granule specific protease.

Experimental Procedures

Preparation of a cDNA Library from Bovine Adrenal Glands. Polyadenylated mRNA from bovine adrenomedullar tissue was prepared by the "low-temperature" modification of the guanidinium thiocyanate method (Han et al., 1987), followed by two cycles of oligo(dT)–cellulose chromatography. A cDNA library was constructed by the Okayama and Berg (1983) method, using a poly(dT)-tailed pCDV1-Pl-SP6 vector primer–linker system (Noma et al., 1986). This procedure was modified slightly to include a denaturation of poly(A) mRNA with 0.01 M methylmercury hydroxide for 10 min at 25 °C, followed by the addition of 0.03 M mercaptoethanol to remove methylmercury hydroxide. cDNA first strand synthesis was carried out in a reaction mixture containing 10 μg of denatured mRNA, 2 μg of vector primer, 40 mM KCl, 8 mM $MgCl_2$, 100 mM Tris-HCl, pH 8.3, 50 μg/mL actinomycin D, 2 mM dNTPs, 20 μCi of [α-^{32}P]dCTP (3000 Ci/mmol), 40 units of avian myeloblastosis reverse transcriptase (diluted 10-fold in 10% glycerol, 10 mM KP_i, pH 7.4, 0.2% Triton X-100, and 2 mM dithiothreitol just before the addition), and 400 units of moloney murine leukemia virus transcriptase. The final reaction volume was 100 μL and was incubated at 40 °C for 1 h. All subsequent steps were performed as described by Okayama and Berg (1983).

Isolation and Characterization of DβM cDNA Clones. The above-described library, consisting of 3×10^5 independent clones, was screened by two antisense oligonucleotides: 5′-CATGTGGTGGACAAGGGCCTCATTGCCCTTGGT-GACGATGGGCTCGTA-3′ and 5′-CATCACTGGCGT-GTACGCCAGTCCAGCTCCAT-3′, corresponding to residues 731–778 and 1079–1111 in the final cDNA sequence, respectively. These oligonucleotide sequences were deduced from previously published peptide fragments (McCafferty & Hogue-Angeletti, 1987) with a bovine codon usage frequency table (Maruyama et al., 1986) to determine the third position of degenerate codons. Restriction fragments of clones giving positive hybridization signals were subcloned to pGEM 7Zf$^+$ and PGEM 7Zf$^-$. Single-stranded DNA was prepared after transfection of the liquid cultures with M13KO7 helper phage at a multiplicity of infection of 20, and both strands of all regions were sequenced by the chain termination method (Sanger, 1977) with modified T-7 DNA polymerase (sequenase from U.S. Biochemical Corp.). Some GC-rich regions of the gene were sequenced with Taq DNA polymerase, 7-deaza-dGTP being used in place of dGTP in the reaction mixture. Hydropathy plot analyses of the final DβM protein sequence were conducted according to Kyte and Doolittle (1982). Homology comparisons between bovine DβM and other proteins were carried with a Sun Micro Systems Intelligenetics DNA analysis package.

Nuclease Protection Experiments. Antisense RNA was synthesized from subclones consisting of the 5′-end (positions 8–291) and 3′-end (positions 1527–1925) of the coding region with SP-6 and T-7 RNA polymerases. In vitro transcription reactions were performed as described by Krieg and Melton (1984). RNase mapping was performed by solution hybridization of 5 μg of total adrenomedullar poly(A) RNA and labeled antisense RNA (10^5 dpm) in 30 μL of buffer containing 80% formamide, 40 mM PIPES, pH 6.8, 0.4 M NaCl, and 1 mM EDTA at 45 °C for 15 h. This was followed by the addition of 300 μL of RNase digestion buffer containing 0.3 M NaCl, 10 mM Tris-HCl, pH 7.5, 5 mM EDTA, 40 μg/mL RNase A, and 2 μg/mL RNase T1. After incubation for 60 min at 30 °C, the reaction was terminated by digestion with 50 μg/mL proteinase K in the presence of 0.5% SDS, followed by phenol–chloroform extraction and ethanol precipitation in the presence of yeast tRNA as carrier. The protected fragments were analyzed in a 5% denaturing acrylamide–8% urea gel.

Isolation of Soluble DβM. Soluble enzyme was prepared from lysates of fresh adrenal medulla as described previously (Stewart & Klinman, 1988; Klinman & Krueger, 1982), except that a second DE-52 column was run to ensure a high level of purity.

Isolation of Membranous DβM. Membranous enzyme was purified by a modification of the procedure of Saxena and Fleming (1983). Highly purified chromaffin granules were obtained from fresh bovine adrenal medulla by centrifugation through 1.6 M sucrose (Bartlett & Smith, 1974). While catalase (100 μg/mL) was included in all sucrose solutions, no phenylmethanesulfonyl fluoride was added to either sucrose or subsequent granule lysis buffers. An aliquot of granules was subjected to lysis in 5 mM KP_i, pH 6.6, containing 100 μg/mL catalase, followed by two cycles of freezing and thawing. The membranes were collected by centrifugation at 100000*g*, resuspended in lysis buffer (minus catalase), and again collected by centrifugation. This process of resuspension and centrifugation was repeated three more times to facilitate removal of as much soluble protein as possible. The final pellet of washed membranes was resuspended in 5 mM KP_i, and the membrane proteins were extracted by stirring at 4 °C in the presence of 2% (w/v) octyl glucoside. After removal of membranes by centrifugation at 35000*g*, the supernatant was diluted to an octyl glucoside concentration of 1% and applied to a DE-52 anion exchange column (1.5 × 20 cm) equilibrated in 5 mM KP_i, pH 6.6, containing 1% octyl glucoside. Fractions containing DβM activity were eluted and concentrated as previously described (Saxena & Fleming, 1983) and applied to a column of Sepharose CL-6B (1.5 × 20 cm) equilibrated in a running buffer of 50 mM MES, pH 6.5, containing 0.1 M NaCl and 1% octyl glucoside. DβM was eluted at a flow rate of 12 mL/h; fractions containing the highest specific activity were concentrated on an Amicon PM-30 membrane. Because the enzyme at this stage was still significantly contaminated by a protein of 55 kDa, which begins to elute at the end of the DβM peak, the concentrate was reapplied to the Sepharose column under the identical conditions described above. Fractions containing a DβM specific activity of >2 units/mg were pooled, concentrated, and rinsed with a 10-mL wash of 50 mM MES, pH 6.5, containing 0.1 M NaCl but no octyl glucoside. The final concentration of isolated membranous DβM was determined by the method of Bradford (1976) using soluble monooxygenase of known concentration (Stewart & Klinman, 1988b; Skotland et al., 1977) as a standard. Approximately 1 mg of membranous dopamine β-monooxygenase was purified from 55 mg of total membrane protein; the final specific activity of the enzyme was 5.2 units/mg in an assay system consisting of 50 mM MES, pH 6.0, 10 mM ascorbate, 10 mM tyramine hydrochloride, 10 mM fumarate, 100 μg/mL catalase, and 2 μM $CuSO_4$. The activity of DβM was assayed as the steady-state rate of oxygen consumption with a polarographic electrode.

Separation of Subunit Bands by Electrophoresis. A 150-μg portion of membranous DβM (heated for 5 min in β-mercaptoethanol-containing sample buffer) was applied to a 3-mm, 7.5% polyacrylamide gel in the presence of 0.1% SDS. Following electrophoresis and Coomassie staining, 75- and 72-kDa band slices were removed and placed in separate Schleicher & Schuell electroelution devices. Protein was eluted with a buffer of 1 M Tris, 200 mM glycine, and 0.1% SDS. Following electroelution, the gel was restained with Coomassie and found to be devoid of protein, indicating close to complete elution of protein bands from the gel. The yield of each band from electroelution can be estimated in the following way: from analytical gel electrophoresis of membrane-derived DβM, the major protein bands were at 75 and 72 kDa with minor (≤20%) contaminants at 69 and 55 kDa. A conservative estimate of the ratio of the 75- to 72-kDa bands indicates a value of 0.35:0.65, for a final yield of 42 μg for the 75-kDa band and 78 μg for the 72-kDa band. Samples were dialyzed against 0.1% SDS, dried, and sequenced.

N-Terminal Sequence Analysis. Samples of both soluble and membranous DβM containing 40–300 μg of protein were precipitated at 4 °C in 10% trichloroacetic acid and the pellets washed with acetone and dried in vacuo. N-Terminal sequence analysis was performed on both Beckman 890M and Applied Biosystems 470A automated sequencers. The quantity of protein applied to the sequencers was confirmed by amino acid analysis on a Beckman 6300 analyzer.

Results

Molecular Cloning and Sequencing of Bovine Adrenal DβM. Initially, a cDNA library was constructed from bovine adrenal medulla poly(A) RNA following the protocols of Okayama and Berg (1983). Screening with the two oligonucleotide probes described under Experimental Procedures yielded a partial cDNA clone of 1.6 kb, which corresponded to the 3′-half of bovine DβM. It should be noted that screening with these oligonucleotide probes was found to yield a very large number of "false positives" and that of ca. 18 positive clones only *one* was found to correspond to DβM. Rescreening with our 1.6-kb probe failed to indicate a full-length clone. A second library was therefore constructed, in which a modified first strand synthesis step was introduced into the Okayama–Berg method. It was found that the combined use of two viral reverse transcriptases and a methylmercury hydroxide mediated RNA denaturation increased the yield of long cDNA inserts in the final library and the efficiency of the first strand synthesis from 15–20% to 25–30%. These modifications were found to be *essential* in obtaining a full-length clone for DβM.

Our second library, consisting of 3×10^5 independent clones, was screened with the 1.6-kb partial clone of DβM obtained from the initial library. After three rounds of screening, 51 clones gave a positive hybridization signal; 20 of these were randomly selected and further characterized by restriction enzyme mapping and 5′-end sequencing. A total of 10 clones were found to be full length, as judged by their insert size (2.4 kb) and the nucleotide sequence at the 5′-ends. The nucleotide sequence of pDβM1 is shown in Figure 1; an identical sequence was found for 8 other clones. One clone differed from this sequence at position 1357, where a TAC triplet replaced the CAG, resulting in a change of Gln to Tyr at position 440 in the polypeptide chain. This difference does not appear to be due to an error in reverse transcriptase, since an identical amino acid substitution was found in two other partial clones. Given the evidence that DβM is a single-copy gene (Kobayashi et al., 1989), we believe this change represents a true allelic polymorphism that could be screened by *Sma*I restriction enzyme mapping.

The first ATG codon was identified as the initiation codon, since it is preceded by a CAGCC consensus sequence (Kozak, 1981); additionally, the N-terminal sequence of membranous DβM starts from this point (see below). It is followed by an open reading frame 1833 bp long, encoding a 610 amino acid polypeptide. Calculated molecular sizes—68.1 kDa for protein containing the signal sequence and 64.8 kDa for soluble enzyme—are consistent with the estimated molecular size of the unglycosylated protein (Speedie et al., 1985). The coding region is followed by a 485-bp 3′-untranslated region. The most common polyadenylation consensus signal, -AATAAA-,

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       10                                        40                                        70
CCCAGCC ATG CAG GTC CCC AGC CCC AGC GTG CGC GAG GCG GCC TCC ATG TAC GGC ACC GCG GTG GCC GTC TTC CTG GTC ATC CTC GTG GCT
        Met Gln Val Pro Ser Pro Ser Val Arg Glu Ala Ala Ser Met Tyr Gly Thr Ala Val Ala Val Phe Leu Val Ile Leu Val Ala  28
       100  ↓                                    130                                       160                    *
GCA CTG CAG GGC TCG GCC CCC GCC GAG AGC CCC TTC CCC TTC CAC ATC CCC CTG GAC CCC GAG GGG ACC CTG GAG CTG TCC TGG AAC ATC
Ala Leu Gln Gly Ser Ala Pro Ala Glu Ser Pro Phe Pro Phe His Ile Pro Leu Asp Pro Glu Gly Thr Leu Glu Leu Ser Trp Asn Ile  58
       190                                       220          I                            250
AGC TAT GCG CAG GAG ACC ATC TAC TTC CAG CTC CTG GTG CGG GAG CTC AAG GCT GGT GTC CTG TTT GGG ATG TCG GAC CGA GGG GAG CTG
Ser Tyr Ala Gln Glu Thr Ile Tyr Phe Gln Leu Leu Val Arg Glu Leu Lys Ala Gly Val Leu Phe Gly Met Ser Asp Arg Gly Glu Leu  88
       280                                       310                                       340
GAG AAT GCT GAC TTG GTG GTG CTC TGG ACT GAC AGG GAC GGC GCC TAC TTT GGG GAT GCC TGG AGT GAC CAG AAG GGG CAG GTC CAC CTG
Glu Asn Ala Asp Leu Val Val Leu Trp Thr Asp Arg Asp Gly Ala Tyr Phe Gly Asp Ala Trp Ser Asp Gln Lys Gly Gln Val His Leu 118
       370                                       400                   II                  430
GAC TCC CAG CAG GAT TAC CAG CTT CTG CGG GCA CAG AGG ACT CCA GAA GGC CTG TAC CTG CTC TTC AAG AGG CCT TTT GGC ACC TGT GAC
Asp Ser Gln Gln Asp Tyr Gln Leu Leu Arg Ala Gln Arg Thr Pro Glu Gly Leu Tyr Leu Leu Phe Lys Arg Pro Phe Gly Thr Cys Asp 148
       460                                       490                                       520                    *
CCC AAC GAC TAC CTC ATC GAG GAC GGC ACC GTC CAC CTG GTG TAT GGA TTC CTG GAG GAG CCG CTC CGG TCG CTG GAG TCC ATC AAC ACA
Pro Asn Asp Tyr Leu Ile Glu Asp Gly Thr Val His Leu Val Tyr Gly Phe Leu Glu Glu Pro Leu Arg Ser Leu Glu Ser Ile Asn Thr 178
       550                                       580                                       610
TCC GGC TTG CAC ACG GGG CTG CAG AGG GTG CAG CTG CTG AAG CCC AGC ATC CCG AAG CCG GCC CTG CCC GCG GAC ACG TGC ACC ATG GAG
Ser Gly Leu His Thr Gly Leu Gln Arg Val Gln Leu Leu Lys Pro Ser Ile Pro Lys Pro Ala Leu Pro Ala Asp Thr Cys Thr Met Glu 208
       640                                       670                                       700
ATC CGC GCC CCC GAC GTC CTC ATC CCC GGC CAG CAG ACC ACG TAC TGG TGC TAC GTG ACC GAG CTC CCG GAC GGC TTC CCC CGG CAC CAC
Ile Arg Ala Pro Asp Val Leu Ile Pro Gly Gln Gln Thr Thr Tyr Trp Cys Tyr Val Thr Glu Leu Pro Asp Gly Phe Pro Arg His His 238
       730                                       760       III                             790
ATC GTC ATG TAC GAG CCC ATC GTC ACC GAG GGC AAC GAG GCG CTG GTG CAC CAC ATG GAG GTC TTT CAG TGC GCC GCC GAG TTT CGA GAC
Ile Val Met Tyr Glu Pro Ile Val Thr Glu Gly Asn Glu Ala Leu Val His His Met Glu Val Phe Gln Cys Ala Ala Glu Phe Arg Asp 268
       820                                       850  IV                                   880
CAT CCC CAC TTC AGC GGG CCC TGC GAC TCC AAG ATG AAG CCG CAG CGG CTC AAC TTC TGC CGT CAC GTG CTG GCC GCC TGG GCC CTG GGC
His Pro His Phe Ser Gly Pro Cys Asp Ser Lys Met Lys Pro Gln Arg Leu Asn Phe Cys Arg His Val Leu Ala Ala Trp Ala Leu Gly 298
       910                                       940                                       970
GCC AAG GCC TTT TAC TAC CCA GAG GAA GCA GGC CTG GCC TTC GGG GGG CCC GGC TCC TCC AGA TTT CTC CGC CTG GAA GTT CAC TAC CAC
Ala Lys Ala Phe Tyr Tyr Pro Glu Glu Ala Gly Leu Ala Phe Gly Gly Pro Gly Ser Ser Arg Phe Leu Arg Leu Glu Val His Tyr His 328
      1000                                      1030                                      1060
AAC CCA CTG GTG ATA ACA GGC CGG CGC GAC TCC TCG GGC ATC CGC CTG TAC TAC ACG GCT GCG CTG CGG CGC TTC GAC GCG GGC ATC ATG
Asn Pro Leu Val Ile Thr Gly Arg Arg Asp Ser Ser Gly Ile Arg Leu Tyr Tyr Thr Ala Ala Leu Arg Arg Phe Asp Ala Gly Ile Met 358
      1090                                      1120                                      1150
GAG CTG GGC CTG GCG TAC ACG CCC GTG ATG GCC ATC CCC CCG CAG GAG ACG GCC TTC GTC CTC ACC GGC TAC TGC ACG GAC AAG TGC ACC
Glu Leu Gly Leu Ala Tyr Thr Pro Val Met Ala Ile Pro Pro Gln Glu Thr Ala Phe Val Leu Thr Gly Tyr Cys Thr Asp Lys Cys Thr 388
      1180                                      1210           V                          1240
CAG CTG GCC CTG CCC GCC TCA GGG ATT CAC ATC TTC GCC TCT CAG CTC CAC ACG CAC CTG ACC GGC CGG AAG GTG GTC ACA GTG CTG GCC
Gln Leu Ala Leu Pro Ala Ser Gly Ile His Ile Phe Ala Ser Gln Leu His Thr His Leu Thr Gly Arg Lys Val Val Thr Val Leu Ala 418
      1270                                VI    1300                                      1330
AGG GAC GGC CGG GAG ACA GAG ATC GTG AAC AGG GAC AAC CAC TAC AGC CCA CAC TTC CAG GAG ATC CGC ATG TTG AAG AAG GTC GTG TCT
Arg Asp Gly Arg Glu Thr Glu Ile Val Asn Arg Asp Asn His Tyr Ser Pro His Phe Gln Glu Ile Arg Met Leu Lys Lys Val Val Ser 438
      1360                                      1390                                      1420
GTC CAG CCG GGA GAC GTG CTC ATC ACC TCT TGC ACA TAC AAC ACG GAA GAC AGG AGG CTG GCC ACC GTG GGG GGC TTC GGG ATC CTG GAG
Val Gln Pro Gly Asp Val Leu Ile Thr Ser Cys Thr Tyr Asn Thr Glu Asp Arg Arg Leu Ala Thr Val Gly Gly Phe Gly Ile Leu Glu 468
      1450                                      1480                                      1510
GAG ATG TGC GTC AAC TAT GTG CAC TAC TAC CCC CAG ACG CAG CTG GAG CTC TGC AAG AGC GCC GTG GAC CCT GGC TTC CTG CAC AAG TAC
Glu Met Cys Val Asn Tyr Val His Tyr Tyr Pro Gln Thr Gln Leu Glu Leu Cys Lys Ser Ala Val Asp Pro Gly Phe Leu His Lys Tyr 498
      1540                                      1570                                      1600
TTC CGC CTC GTG AAC AGG TTC AAC AGC GAG GAA GTC TGC ACC TGC CCC CAG GCG TCT GTC CCT GAG CAG TTT GCC TCC GTG CCC TGG AAC
Phe Arg Leu Val Asn Arg Phe Asn Ser Glu Glu Val Cys Thr Cys Pro Gln Ala Ser Val Pro Glu Gln Phe Ala Ser Val Pro Trp Asn 528
      1630                                      1660                                  *   1690
TCC TTC AAC CGC GAG GTG CTC AAG GCC CTG TAC GGC TTC GCA CCC ATC TCC ATG CAC TGC AAC AGG TCC TCG GCC GTC CGC TTC CAG GGC
Ser Phe Asn Arg Glu Val Leu Lys Ala Leu Tyr Gly Phe Ala Pro Ile Ser Met His Cys Asn Arg Ser Ser Ala Val Arg Phe Gln Gly 558
      1720                                      1750                                      1780                   VII
GAG TGG AAT CGG CAG CCC CTG CCT GAG ATC GTG TCC AGG TTG GAA GAG CCC ACC CCT CAG TGC CCA GCC AGC CAG GCT CAG AGC CCC GCC
Glu Trp Asn Arg Gln Pro Leu Pro Glu Ile Val Ser Arg Leu Glu Glu Pro Thr Pro Gln Cys Pro Ala Ser Gln Ala Gln Ser Pro Ala 588
      1810          *                           1840
GGC CCC ACC GTG CTG AAC ATC AGT GGG GGC AAA GGC TGAACGTGGGCAGTGTCCTCGCTCCCCTCACCATGCTGTCCTGCGGGCTCACAGCAGCCCTGTGCACCCCC
Gly Pro Thr Val Leu Asn Ile Ser Gly Gly Lys Gly OP
TACTCTGTGAAGACCCCCATGGAATAGCCCAGCACGGAGGGCTGGACCAAGCCACCACCTGAGACCAGGGTCCGGTCCAGCTTTCTCCCCCCAGGGTCCCCCTGCATGGCTGTGACCCT
CCCGTGACAACTTTTGCTGACCCACCGAGGCCCAGGTGGACCAGGACCCTTGCACACCCTTTGACACAGCATAAGAGCAACCCCTTTTGGAAGTCTAGAGTCCAGAGCCCCGAGAGCCC
TGCCATCTCGCTGGGGCTGGGGGGTGCCCGTCTCCTGGGACACGAGCCACGACCGGACCCCGGCCAGACTCCCGGAGCTCTCCCGGCCCCGCGTCCCCAGCCCCCACGGAGGGTGATCG
GTGTTGAGTGTGACGGGTGCAAGTGCTGTTGTACTTAAATGTGTCCCTGCAGACCGG - A63
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FIGURE 1: Nucleotide sequence of the cDNA clone encoding bovine DβH. Nucleotides are numbered in the 5′- to 3′-direction. Numbering of the amino acid positions—right margin—begins with the first methionine. Amino acid residues underlined correspond to the following: peptide I, from N-terminal sequencing (Joh & Hwang, 1986); peptides II, IV, V, and VII, from sequencing of tryptic and cyanogen bromide cleavage products (McCafferty & Hogue-Angeletti, 1987); peptides III and VI, from sequencing of active site labeled peptides (DeWolf et al., 1988, 1989). The asparagine residues labeled with asterisks represent potential N-linked glycosylation sites. The arrow at Gly-32 indicates the putative signal peptide cleavage site.

is not present in the sequence, with the closest match to this signal, -CTTAAA-, found 24 nucleotides upstream of the polyadenylation site.

To establish the authenticity of pDβM1, its deduced amino acid sequence was compared to peptide sequences of the native enzyme. As shown in Figure 1, peptide sequence I corresponds exactly to the N-terminus of soluble enzyme (Joh & Hwang, 1986). Additionally, peptides III and VI, available from the labeling of bovine DβM by mechanism-based inhibitors (DeWolf et al., 1988, 1989), and peptides II, IV, V, and VII, available from tryptic and cyanogen bromide cleavage of DβM (McCafferty & Hogue-Angeletti, 1987), show identity to the cDNA-derived sequence. Comparison of the sequence of the polypeptide for soluble enzyme also indicate an 81% overall homology with human enzyme (Lamouroux et al., 1987). The entire primary structure contains four asparagine residues that are part of the consensus sequence Asn-X-Ser/Thr for N-linked glycosylation sites. The report of six glycosyl moieties per tetramer (Margolis et al., 1984) indicates that only some of these sites are acted upon by glycosylating enzymes. The

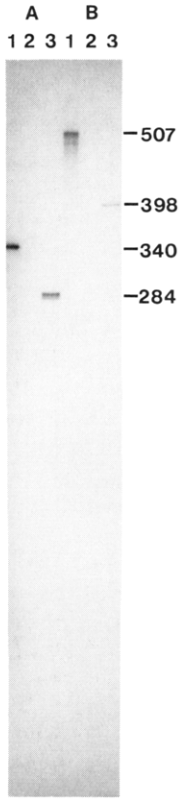

FIGURE 2: RNase protection analysis of the 5′- and 3′-ends of bovine DβH RNA. Polyadenylated RNA from bovine adrenal medulla was protected with antisense RNA probes complementary to the 5′-end (panel A) and 3′-end (panel B) of clone pDβH1 as described under Experimental Procedures. Lanes 1, undigested probe; lanes 2, yeast tRNA control protection, lanes 3, adrenomedullar poly(A) RNA protection. The numbers to the right indicate the length in nucleotide bases.

hydropathy plot of the cDNA-derived DβM polypeptide revealed a single potential hydrophobic anchor segment spanning amino acids 18–30. This result is similar to results seen previously with DβM cloned from a human source, in which a single hydrophobic segment was found to be located at the N-terminus (Lamouroux et al., 1987).

Since DβM exists in both a soluble and membrane-bound form, the occurrence of multiple mRNAs, generated by alternative splicing, has been considered. In the case of human DβM, S1 nuclease protection experiments (Lamouroux et al., 1987) supported a single mRNA species for the coding region. In the present study, restriction enzyme mapping and partial sequencing of 10 independent clones indicate identical sequences with the exception of position 450 (see above). In addition, analysis of bovine adrenomedullar RNA by northern blot hydridization showed a single band at 2.4 kb (data not shown), ruling out the presence of alternative messages of different lengths. As a final test for the existence of multiple RNAs, RNase protection experiments were carried out on bovine adrenal medulla RNA using RNA probes covering the regions of nucleotides 8–291 (284 bases) at the 5′-end and 1527–1925 (398 bases) at the 3′-end of the gene (see Experimental Procedures). As shown in Figure 2, both probes protected a single fragment of the expected size. Thus, we conclude that a single mRNA exists for bovine DβM and, hence, that the generation of dual enzyme forms does not occur at the transcriptional level.

Sequencing of Soluble and Membrane-Derived DβM. Both the soluble and membrane-derived forms of DβM contain two major bands of 75 and 72 kDa (Saxena & Fleming, 1983).

Table I: N-Terminal Sequencing of Membrane-Derived Dopamine β-Monooxygenase[a]

	sequence				sequence		
cycle	A	B	C	cycle	A	B	C
1	Ser	Ala	Met	15	Asp	Gly	Tyr
2	Ala	Glu	Gln	16	Pro	(Thr)	Gly
3	Pro	Ser	Val	17	Glu	Leu	Val
4	Ala	Pro	Phe	18	Gly	Glu	Ala
5	Glu	Phe	Ser	19	(Thr)	Leu	Val
6	Ser	Pro	Val	20	Leu	Ser	Ala/Phe[b]
7	Pro	Phe	Ser	21	Glu	Val	(Asp)
8	Phe	His	Val	22	Leu		Phe
9	Pro	Ile	Arg	23	(Ser)	Ile	Leu
10	Phe	Pho	Glu	24	Val	Gly	(Val)
11	His	Leu	Ala	25		Tyr	Ile
12	Ile	Asp	Ala	26	Ile	Ala	Leu
13	(Ser)	Pro	Phe	27		Gln	Val
14	Leu	Glu	Met				

[a] Sequences A and B were present at approximately the 100-pmol level, and sequence C was present at the 80-pmol level. Residues in parentheses are unconfirmed identifications. [b] Four amino acids were identifiable in this cycle.

While the soluble protein contains a much greater proportion of the 72-kDa subunit, the membranous enzyme is consistently found to be enriched in the heavier subunit. Dhawan et al. (1987) have reported that the membranous enzyme is a heterotetramer comprised of half membrane-attached and half soluble subunits, implying a ratio of *at least* 1:1 for the 75-kDa:72-kDa subunits. However, we routinely find preparations of membrane-derived DβM to be somewhat depleted in the 75-kDa band, suggesting that processing of the heavy subunit may occur during isolation of the membrane-derived subunit and that the soluble subunit is very hard to remove from the mixture.

Samples of purified preparations of soluble and membrane-bound enzymes (each containing a mixture of two subunits) were subjected to N-terminal sequencing. The soluble form of DβM was found to contain two N-terminal sequences (data not shown), corresponding to those previously reported (Joh & Hwang, 1986; Taylor et al., 1989). Because the difference between these two sequences is only three amino acids at the N-terminus, the difference between the 75- and 72-kDa subunits of soluble DβM is unlikely to be at the polypeptide level. It could be argued that the 75-kDa soluble subunit contains a blocked N-terminus and that the sequences obtained represent the 72-kDa form. However, the high recoveries of these sequences relative to the determined amount of protein from amino acid analysis (data not shown) make this very unlikely. Both Speedie et al. (1985) and Oyarce and Fleming (1988) have, in fact, shown that treatment of the soluble enzyme form of DβM with glycosidases results in the complete conversion of the 75- and 72-kDa subunits to a 69-kDa form, implying that different patterns of glycosylation are the cause of size heterogeneity in the soluble enzyme.

As summarized in Table I, gas-phase sequencing of the purified membranous enzyme (again containing a mixture of 75- and 72-kDa subunits) was more complex, revealing the presence of a mixture of three polypeptide chains. An examination of this mixture identified the presence of the two sequences previously obtained from the soluble form of DβM (sequences A and B, Table I). The remaining amino acid in each cycle was thus assigned to sequence C (Table I). Although in many cycles this third amino acid was the least abundant, it was possible to identify clearly this "difference" sequence in the majority of cases.

In order to confirm sequence C and to demonstrate that this sequence arises solely from the 75-kDa subunit of membranous

```
                          *       *                           *
          1   2   3   4   5   6   7   8   9   10  11  12  13
cDNA:    Met Gln Val Pro Ser Pro Ser Val Arg Glu Ala Ala Ser
Protein: Met Gln Val Phe Ser Val Ser Val Arg Glu Ala Ala Phe
                      *               *
         14  15  16  17  18  19  20  21  22  23  24  25  26  27
        Met Tyr Gly Thr Ala Val Ala Val Phe Leu Val Ile Leu Val
        Met Tyr Gly Val Ala Val Ala Asp Phe Leu Val Ile Leu Val
```

FIGURE 3: Alignment of the N-termini of bovine DβM, deduced from cDNA and protein sequencing. The five sites of mismatch are labeled with asterisks.

Table II: N-Terminal Sequencing of Isolated 75- and 72-kDa Subunits from Membrane-Derived DβM

	72 kDa[a]		75 kDa[b]		
cycle	A	B	A	B	C
1	Ser	Ala	Ser	Ala	Met
2	Ala	Glu	Ala	Glu	Gln
3	Pro	Ser	Pro	Ser	Val
4	Ala	Pro	Ala	Pro	Phe
5	Glu	Phe	Glu	Phe	Ser
6	Ser	Pro	Ser	Pro	Val
7			Pro	Phe	Ser
8			Phe	His	Val
9			Pro	Ile	Arg

[a] Sequences A and B of the 72-kDa band were present in equimolar amounts at the level of 50 pmol. [b] Sequence C of the 75-kDa subunit constituted approximately 70% of the total sequence recovered and was present at the level of ~30 pmol. The protein isolated from this band was sequenced through 14 cycles and found to match exactly with the sequence data of Table I.

DβM, the 72- and 75-kDa subunits of membrane-derived enzyme were separated by preparative SDS/PAGE, purified by electroelution, and sequenced from the N-terminus. As seen in Table II, the 72-kDa band of membrane-derived DβM contains two sequences (A and B) in equimolar amounts which are identical with those obtained from sequencing of the soluble enzyme. The 75-kDa band was found, once again, to contain three amino acids at each cycle; *however, sequence C is now present as the majority sequence, constituting ~70% of the total sequence recovered.* The presence of low levels of s/r sequences A and B in the 75-kDa band may be due to a small amount of contamination by the 72-kDa band or, more likely, to the presence of some of the 75-kDa band of the soluble enzyme in membranous preparations.

These overall findings have now been confirmed in separate samples of membrane-derived DβM, leading us to conclude that the 75-kDa subunit of membrane-derived enzyme contains an N-terminus that is completely distinct from that of the 72- and 75-kDa subunits of soluble enzyme.

Discussion

As summarized in Figure 3, the homology between the N-terminus of sequence C of the 75-kDa subunit of membrane-derived DβM with that deduced from cDNA sequencing is striking. Of a total of the first 27 residues, 22 show identity. In four of the remaining five positions (4, 6, 13, and 21), the amino acid coded by the cDNA sequence is also present in the A or B sequence of the membranous enzyme, making a definitive assignment to sequence C difficult. Given the observation of identical signal sequences among 10 independent clones, differences at these positions are most likely due to ambiguities arising from the sequencing of protein mixtures [cf. Blumenfeld et al. (1987)]. *This comparison indicates that the heavy subunit of membranous enzyme has retained its N-terminal signal peptide.*

These results are in disagreement with those reported by Taylor et al. (1989), who found almost identical N-terminal sequences for both the soluble and membrane-derived forms of DβM. Examination of their sequence data, however, does reveal a small extension (by three amino acids) at the N-terminus of membrane-derived DβM. This suggests that proteolytic cleavage may have occurred in their preparations, leading to a partially processed form of membranous DβM. In light of the data presented herein, we suggest that alterations in purification procedures may lead to contamination of membranous DβM preparations by a chromaffin granule specific protease.

Increasingly, there are examples of membrane-associated proteins that undergo anchoring via uncleaved signal peptides. While DβM provides the first example of such anchoring for a secretory vesicle protein, examination of its signal peptide reveals three structurally and possibly functionally distinct regions. These include (1) a hydrophilic NH_2 terminus, comprised of residues 1–17, (2) a hydrophobic core, residues 18–30, and (3) a polar CO_2H-terminal region. This structure satisfies reasonably well the criteria proposed for the three domains of signal peptides (Von Heijne, 1983).

It is well-known that the major portion of the DβM subunit (membranous as well as soluble) faces the interior of chromaffin vesicles [cf. Stewart and Klinman (1988a)]. Thus, its N-terminus is expected to reside in the cytoplasm, identifying DβM as a class II membrane-anchored protein. Other examples of this class of membrane-anchored proteins include rat liver asialoglycoprotein (Spiess & Lodish, 1986), human Na^+–K^+ ATPase β-subunit (Kawakami & Nagano, 1988), human transferrin receptor (Zerial et al., 1986), influenza virus neuraminidase (Bos et al., 1984), rat kidney γ-glutamyl transpeptidase (Laperche et al., 1986), and the invariant chain of class II histocompatibility antigens (Lipp & Dobberstein, 1986). Although all of the above proteins (with the exception of kidney γ-glutamyl transpeptidase) exist with their CO_2H termini in the exoplasm, exocytotic fusion of chromaffin granules with the cell membrane will lead to a similar orientation for DβM.

Examination of the membrane anchors of the above-summarized class II proteins reveals a long hydrophobic core (at least 20 amino acids), whereas the DβM signal peptide hydrophobic core consists of only 13 amino acids. A shortened hydrophobic core is seen more often in cleavable signal peptides, which average 10 ± 3 residues (Von Heijne, 1983). This property of DβM may relate to the fact that it exists in *both* a soluble and a membrane-bound form, such that ca. 50% of mature enzyme subunits have undergone proteolytic processing. In this context we note the close proximity of the Gly-32 cleavage site of the DβM signal peptide to its hydrophobic core—much closer than the five- to seven-residue span normally seen in cleavable signal peptides (Von Heijne, 1983). Additionally, while signal peptidases are not known to cleave after proline, DβM undergoes a second cleavage between Pro-35 and Ala-36. These features suggest a role for a unique protease, with properties different from those seen in the better characterized signal peptidases.

The hypothesis of a unique proteolytic enzyme for the generation of soluble DβM has, in fact, been advanced pre-

viously. As noted in the introduction, Sabban et al. (1983) reported that the soluble (73 kDa) subunit of rat DβM is posttranslationally derived from an initially synthesized 77-kDa subunit. Since treatment of rat PC12 cells with either monensin [to block formation of secretory vesicles (Kuhn et al., 1986)] or chloroquine [to disrupt the acidic granular interior (Sabban et al., 1987)] results in a more complete conversion of the 77-kDa subunit to the 73-kDa subunit, enzymatic processing of membranous DβM has been proposed to occur primarily in the late Golgi and immature granules. One preliminary report of a chromaffin granule enzyme capable of converting bovine membranous DβM to its soluble form upon granule lysis at neutral pH has appeared in the literature (Helle et al., 1984). It should be noted, however, that models invoking late posttranslational processing are somewhat difficult to reconcile with the existence of a heavier (75 kDa) glycosylated subunit for soluble bovine DβM, emphasizing the need for further studies to localize and characterize the protease responsible for the production of soluble DβM.

Comparison of the cDNA-derived protein sequence for soluble bovine DβM to that of the human enzyme (Lamouroux et al., 1987) indicates an 81% overall homology. With regard to the signal peptide region, we find the bovine signal peptide to be seven amino acids longer than its human counterpart. Beginning with position 8, however, the remaining 24 amino acids show a high level of conservation with only three changes detected at positions 13, 16, and 21 (leading to an overall homology of 88%). This suggests that species differences exist for the length of the signal peptide in membranous DβM. It is anticipated that a combination of N-terminal sequencing of membrane-derived DβM from other species, as well as the availability of further cDNA sequences, will clarify this point.

It has been suggested that the catecholamine-synthesizing enzymes may be members of a multigene superfamily, on the basis of the cross-reactivity of anti-tyrosine hydroxylase antibodies to DβM and phenylethanolamine *N*-methyltransferase (Joh et al., 1983). However, an examination of overall and regiospecific homologies between our bovine DβM sequence and bovine tyrosine hydroxylase and phenylethanolamine *N*-methyltransferase sequences has failed to reveal any signficant overlap. This finding, which rules out a recent common ancestral gene for bovine DβM and tyrosine hydroxylase, is consistent with the very different active site properties of the two enzymes, such that DβM utilizes copper as a redox cofactor whereas tyrosine hydroxylase employs pterin.

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Coenzyme F_{430} as a Possible Catalyst for the Reductive Dehalogenation of Chlorinated C_1 Hydrocarbons in Methanogenic Bacteria†

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ABSTRACT: Corrinoids, such as aquocobalamin, methylcobalamin, and (cyanoaquo)cobinamide, catalyze the reductive dehalogenation of CCl_4 with titanium(III) citrate as the electron donor [Krone et al. (1989) *Biochemistry 28*, 4908–4914]. We report here that this reaction is also effectively mediated by the nickel-containing porphinoid, coenzyme F_{430}, found in methanogenic bacteria. Chloroform, methylene chloride, methyl chloride, and methane were detected as intermediates and products. Ethane was formed in trace amounts, and several as yet unidentified nonvolatile compounds were also generated. The rate of dehalogenation decreased in the series of CCl_4, $CHCl_3$, and CH_2Cl_2. With coenzyme F_{430} as the catalyst, the reduction of CH_3Cl to CH_4 proceeded more than 50 times faster than with aquocobalamin. Cell suspensions of *Methanosarcina barkeri* were found to catalyze the reductive dehalogenation of CCl_4 with CO as the electron donor ($E'_0 = -0.524$ V). Methylene chloride was the main end product. The kinetics of $CHCl_3$ and CH_2Cl_2 formation from CCl_4 were similar to those with coenzyme F_{430} or aquocobalamin as catalysts and titanium(III) citrate as the reductant.

Various methanogenic bacteria catalyze the reductive dehalogenation of chlorinated hydrocarbons, such as CCl_4, $CHCl_3$, and perchloroethylene (Egli et al., 1987; Belay & Daniels, 1987; Fathepure & Boyd, 1988a,b). It has been suggested that coenzyme F_{430} is involved in the catalysis of these reactions (Fathepure & Boyd, 1988a,b). Coenzyme F_{430} is a nickel(II) porphinoid (Figure 1) present in all methanogenic bacteria (Diekert et al., 1981). It functions as the prosthetic group of methyl coenzyme M (CoM)[1] reductase, which mediates the final step in methanogenesis (Rouvière & Wolfe, 1988; Ellermann et al., 1988, 1989).

$$CH_3S\text{-}CoM + HS\text{-}HTP \rightarrow CH_4 + CoM\text{-}S\text{-}S\text{-}HTP$$

Electron paramagnetic resonance (EPR) spectroscopic studies indicate that the nickel of enzyme-bound coenzyme F_{430} may undergo redox changes (Albracht et al., 1986, 1988; Jaun & Pfaltz, 1986). In vitro Ni(I)F_{430} [Ni(II)F_{430} reduced with zinc amalgam in dimethylformamide] reacts with methyl iodide to yield methane (Jaun & Pfaltz, 1986, 1988).

$$CH_3I + 2Ni(I)F_{430} + H^+ \rightarrow CH_4 + 2Ni(II)F_{430} + I^-$$

Ni(I)F_{430} is oxidized to Ni(II)F_{430} within the mixing time. In vivo methyl iodide (Laufer et al., 1986) and other halogenated C_1 hydrocarbons (CCl_4, $CHCl_3$, and CH_2Br_2) (Bauchop, 1967; Wolfe, 1971; Belay & Daniels, 1987) are known to specifically inhibit methanogenesis from methyl-CoM, probably by oxidizing the reduced F_{430} bound to methyl-CoM reductase.

We have recently reported that corrinoids catalyze the rapid reductive dehalogenation of CCl_4 to CH_3Cl with titanium(III) citrate in aqueous solution at pH 8.2 (Krone et al., 1989). Evidence is presented here that coenzyme F_{430} is an even more effective dehalogenation catalyst capable of reducing CCl_4 to CH_4.

Materials and Methods

Coenzyme F_{430} ($\epsilon_{430} = 22\,500$ M^{-1} cm^{-1}) was isolated from H_2/CO_2-grown *Methanobacterium thermoautotrophicum* (strain Marburg) (Diekert et al., 1981). Aquocobalamin ($\epsilon_{527} = 8500$ M^{-1} cm^{-1}) (Friedrich, 1975), dithioerythritol, and dithiothreitol were obtained from Sigma (München, FRG). Carbon tetrachloride, methylene chloride, and titanium(III) chloride were from Merck (Darmstadt, FRG). Chloroform was from Baker Chemicals (Deventer, The Netherlands), and methyl chloride, methane, and ethane were from Messer Griesheim (Düsseldorf, FRG). Titanium(III) citrate solutions, pH 8, were prepared as described by Zehnder and Wuhrmann

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[1] Abbreviations: B_{12a}, cob(III)alamin; B_{12r}, cob(II)alamin; B_{12s}, cob(I)alamin; CoM, coenzyme M; HS-CoM, 2-mercaptoethanesulfonate; CH_3S-CoM, 2-(methylthio)ethanesulfonate; CoM-S-S-HTP, mixed disulfide of HS-CoM and HS-HTP; HS-HTP, *N*-(7-mercaptoheptanoyl)-threonine phosphate; Tris, tris(hydroxymethyl)aminomethane.

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