The EMBO Journal vol.6 no.13 pp.3931-3937, 1987

The primary structure of human dopamine-β-hydroxylase: insights into the relationship between the soluble and the membrane-bound forms of the enzyme

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Communicated by J.-P.Changeux

A full length dopamine-β-hydroxylase (DBH) cDNA clone was isolated from a human pheochromocytoma λgt11 library. Both structural and functional evidence confirms the authenticity of the clone: (i) antibodies selected with fusion proteins generated by positive clones precipitate DBH activity, (ii) the sequence of three internal DBH tryptic peptides are included in the deduced DBH sequence, (iii) the previously reported N-terminal 15 amino acids of bovine DBH exhibits a nearly complete identity with that predicted for human DBH. The polypeptide chain of DBH comprises 578 amino acids corresponding to an unmodified protein of 64 862 daltons and is preceded by a cleaved signal peptide of 25 residues. DBH exists in both membrane-bound and soluble forms. The hydropathy plot reveals no obvious hydrophobic segment, except the signal peptide. S1 mapping analysis indicates no diversity in the 5′ and 3′ extremities of the DBH mRNA. Taken together with available biochemical data, these observations suggest that the membrane attachment of DBH probably results from a post-translational modification, glypiation being the most likely candidate. Comparative amino acid sequence analysis establishes that DBH shares no homology with the other catecholamine synthesizing enzymes, tyrosine hydroxylase and phenylethanolamine-*N*-methyl transferase.

Key words: cDNA sequence/dopamine-β-hydroxylase/human pheochromocytoma/membrane attachment/S1 mapping

Introduction

Dopamine-β-hydroxylase (DBH) (EC 1.14.17.1), the enzyme that converts dopamine into noradrenaline (Levin *et al.*, 1960; Friedman and Kaufman, 1965), belongs, with tyrosine hydroxylase (TH) and phenylethanolamine-*N*-methyl transferase (PNMT), to a family of enzymes involved specifically in the synthesis of catecholamines from tyrosine. The three possible phenotypes of catecholaminergic cells (dopaminergic, noradrenergic and adrenergic) are characterized by the expression of these enzymes; DBH is expressed in noradrenergic and adrenergic cells but not in dopaminergic cells. Thus the catecholamine biosynthesis pathway provides a convenient model to investigate the cell specificity and the coordination of the expression of multiple genes. Moreover, the expression of DBH and TH appears to be co-regulated in various experimental situations. For instance, reserpine (Molinoff *et al.*, 1970; Ciaranello *et al.*, 1975), nerve growth factor (NGF) (Thoenen *et al.*, 1971; Acheson *et al.*, 1984) and neural stimulation (Axelrod, 1972), specifically increase the activity of these two enzymes by increasing their synthesis. It has also been proposed that TH, DBH and PNMT derive from a common ancestral gene (Joh *et al.*, 1983; Baetge *et al.*, 1986).

DBH is characterized by its association with the secretory vesicles in chromaffin cells (Laduron, 1975) and in the noradrenergic nerve endings of the peripheral and central nervous system (Potter and Axelrod, 1963; De Potter *et al.*, 1970; Hartman and Udenfriend, 1972), whereas TH and PNMT are cytoplasmic (Sabban and Goldstein, 1984). Therefore, its study offers an opportunity for approaching the structural features that are involved in the sorting of proteins to the secretory granules. In particular, it would be of interest to know whether DBH shares the common motive found in chromogranin A and secretogranin I (chromogranin B) (Benedum *et al.*, 1987), two other major proteins from the granule matrix.

The intragranular enzyme is present in a soluble and in a membrane-bound form in similar amounts (Belpaire and Laduron, 1968; Winkler *et al.*, 1970), the soluble form being released from the cell by exocytosis (Viveros *et al.*, 1968; Weinshilboum *et al.*, 1971). Both forms are tetrameric copper glycoproteins (Wallace *et al.*, 1973); they have similar immunoreactivities Slater *et al.*, 1981), carbohydrate contents (Fischer-Colbrie *et al.*, 1982) and kinetic parameters (Saxena and Fleming, 1983). SDS gel electrophoresis reveals two different subunits of 77 000 and 73 000 daltons (Saxena and Fleming, 1983; Sabban *et al.*, 1983; Gavine *et al.*, 1984), the larger being thought to be responsible for the attachment of the enzyme to the membrane (Dhawan *et al.*, 1987). Although the relationship between the two forms has been extensively studied, the precise molecular differences, as well as their functional significance, have not been resolved (for a review see Winkler *et al.*, 1986). As a first step towards answering these questions, we report here the isolation of a cDNA clone containing the complete coding sequence of this enzyme.

Results

Cloning of human DBH cDNA

A cDNA expression library was constructed using mRNA extracted from a human pheochromocytoma tumour expressing high levels of DBH activity. The specificity of the anti-human DBH serum has been discussed previously (Antreassian *et al.*, 1986) and for our purpose, its suitability for screening the library was checked on Western blots of purified human DBH and pheochromocytoma homogenates. Out of 75 000 clones examined, 48 were isolated after three rounds of purification. They were all shown to cross-hybridize and six were analysed by restriction mapping and partial sequencing. The longest one, ~ 1300 bp, designated DBH1, was used to screen a λgt10 size-selected library (described previously in Grima *et al.*, 1987), generated from the same pheochromocytoma tumour mRNAs, in order to obtain a complete DBH clone. Out of 50 clones selected, five (DBH2-DBH6, containing inserts of 2.9, 2.8, 2.5, 2.0 and 1.8 kb respectively) were retained for sequence analysis.

Inhibition of DBH activity by affinity-purified antibodies

Inserts of DBH3 and DBH4 were subcloned into λgt11 and the corresponding fusion proteins were prepared and used to select

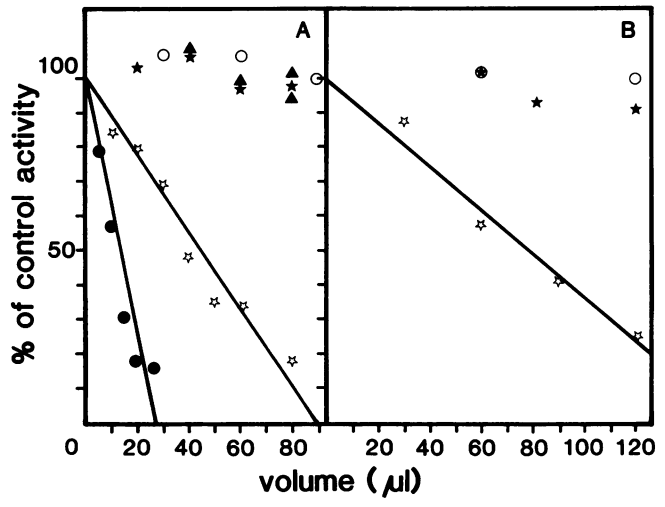

Fig. 1. Inhibition of DBH activity after incubation by various immunoadsorbed antibodies: (**A**) with addition of protein A−Sepharose; (**B**) without addition of protein A−Sepharose. Purified human DBH was incubated with immunoglobulins selected from anti-DBH serum as described in Materials and methods with either fusion protein DBH3 (☆), purified DBH (●), or β-galactosidase (★). Purified DBH was also incubated in the same conditions with the elution buffer alone (▲), or with γ-globulin (3 mg/ml) in PBS containing 1.5 mg/ml BSA (○).

specific antibodies from the anti-human DBH serum. These antibodies were tested for their ability to inhibit DBH activity. Figure 1 presents the results of a typical experiment: both immunoprecipitation (A) and immunotitration (B) of DBH activity were obtained. Similar inhibition curves were produced by using the DBH4 fusion protein instead of that of DBH3. Antibodies selected by the same procedure, with an amount of pure DBH equivalent to that present in the fusion protein (200 µg), were only three times more effective in inhibiting enzyme activity. This difference presumably results from the absence of the NH_2 terminal part of DBH in the fusion protein, as well as from the absence of glycosylation and/or from differences in the tertiary structure of the fusion protein induced by the presence of the β-galactosidase moiety. No inhibition was observed with the dialysed elution buffer alone, with purified non-specific rabbit IgG, or with the eluate of the anti-DBH serum adsorbed on purified β-galactosidase. In a further control experiment performed with a different fusion protein (tryptophan hydroxylase, Darmon *et al.*, 1986) the eluate was unable to inhibit DBH activity when used in the same test (data not shown). Identical results were obtained by testing the selected antibodies on human pheochromocytoma homogenates (results not shown).

Sequence analysis of human DBH tryptic peptides

In parallel to the selection of DBH antibody with a fusion protein, we sought to obtain the sequence of peptides generated from human DBH to compare them to the DBH2 sequence. The enzyme preparation was ascertained to be pure by the following criteria. (i) SDS−PAGE in the presence of β-mercaptoethanol, yielded a major band at 74 000 daltons (Saxena and Fleming, 1983). (ii) In the absence of a reducing agent, only one band of 150 000 daltons was observed (Saxena and Fleming, 1983). (iii) After deglycosylation with *N*-glycanase (Genzyme), the 74 000-dalton band shifted to a 61 000-dalton species (Sabban *et al.*, 1983). (iv) Two-dimensional gel electrophoresis showed a major spot characteristic of DBH preparations with an isoelectric point in the range 6.0−6.5 (Gavine *et al.*, 1984).

Accordingly, the 74 000-dalton band was cut out, eluted and subjected to tryptic digestion; the resulting peptides were separated by HPLC and the N-terminal sequence of three well-separated peaks was determined to be as follows: peptide I, Asp-Tyr-Leu-Ile-Glu-Asp-Gly-Thr-Val-X-Leu-Val; peptide II, Ala-Phe-Tyr-Tyr-Pro-Glu-Glu-Ala-Gly-Leu-Ala; and peptide III, Tyr-Leu-His-Leu-Ile-Asn-Gly.

Nucleotide and deduced amino acid sequence of human DBH

The complete nucleotide sequence of the coding region of clone DBH2 is shown in Figure 2. This clone appears to be nearly full-length, as it contains a poly(A) tail at its 3′ extremity and since a primer extension experiment, performed both with human pheochromocytoma and adrenal gland RNAs, indicates that the initiation of transcription occurs no more than 16 bp upstream from the 5′ extremity of this clone (data not shown). The first ATG codon, beginning at nucleotide 40, is preceeded by the nucleotides C, A and C at positions −4, −3 and −1 respectively; this fits well with the consensus sequence of Kozak (1984), suggesting that this AUG serves as the *in vivo* initiation codon. It opens a reading frame of 1812 bp specifying a 603-amino acid protein. This deduced amino acid sequence includes the previously determined N-terminal sequence (underlined in Figure 2) of each of the three internal tryptic peptides analysed. Only two amino acids differ from those expected: in peptide III, Phe and Arg are found instead of Leu and Gly respectively. Each of these differences can be explained by a single base-pair substitution and might reflect a true polymorphism of the protein, although inaccuracies in the determination of the peptide sequence cannot be completely excluded. The fact that the same sequence was found in three independent clones rules out the possibility of reverse transcriptase errors during the cloning procedure.

The first 26 residues are highly hydrophobic and constitute a typical signal sequence responsible for targeting the nascent polypeptide to the membrane of the rough endoplasmic reticulum. The initial hydrophobic stretch ends with the sequence Gly-Ser-Ala; any of these amino acids could serve as a suitable cleavage site for signal peptidase (Von Heijne, 1984). The fifteen N-terminal amino acids of bovine soluble DBH (Skotland *et al.*, 1977, modified by Joh and Hwang, 1986) align with the human sequence from Ser-26 onwards with only three differences in 15 residues (Figure 3). This comparison indicates that the likely cleavage site of the signal peptide is after Gly-25. Moreover, these data provide supplementary evidence that this clone encodes human DBH.

Throughout the DBH open reading frame, codon usage is highly biased towards the more favoured codons in man, in agreement with the gene's high level of expression (Grantham *et al.*, 1986).

The mol. wt calculated from the DBH2 clone is 67 527 daltons including the signal peptide and 64 862 daltons in the mature form. A recent determination by the technique of radiation inactivation suggesting a value of 123 000 ± 14 000 daltons for the DBH dimer (Gasnier *et al.*, 1987) is in agreement with the present evaluation. The calculated isoelectric point is 6.54. The deduced amino acid composition is consistent with the previously determined compositions of rat (Okuno and Fujisawa, 1984), bovine (Slater *et al.*, 1981; Craine *et al.*, 1973; Foldes *et al.*, 1973; Ljones *et al.*, 1976; Hörtnagl *et al.*, 1972) and human (Stone *et al.*, 1974; Sokoloff *et al.*, 1985) DBH.

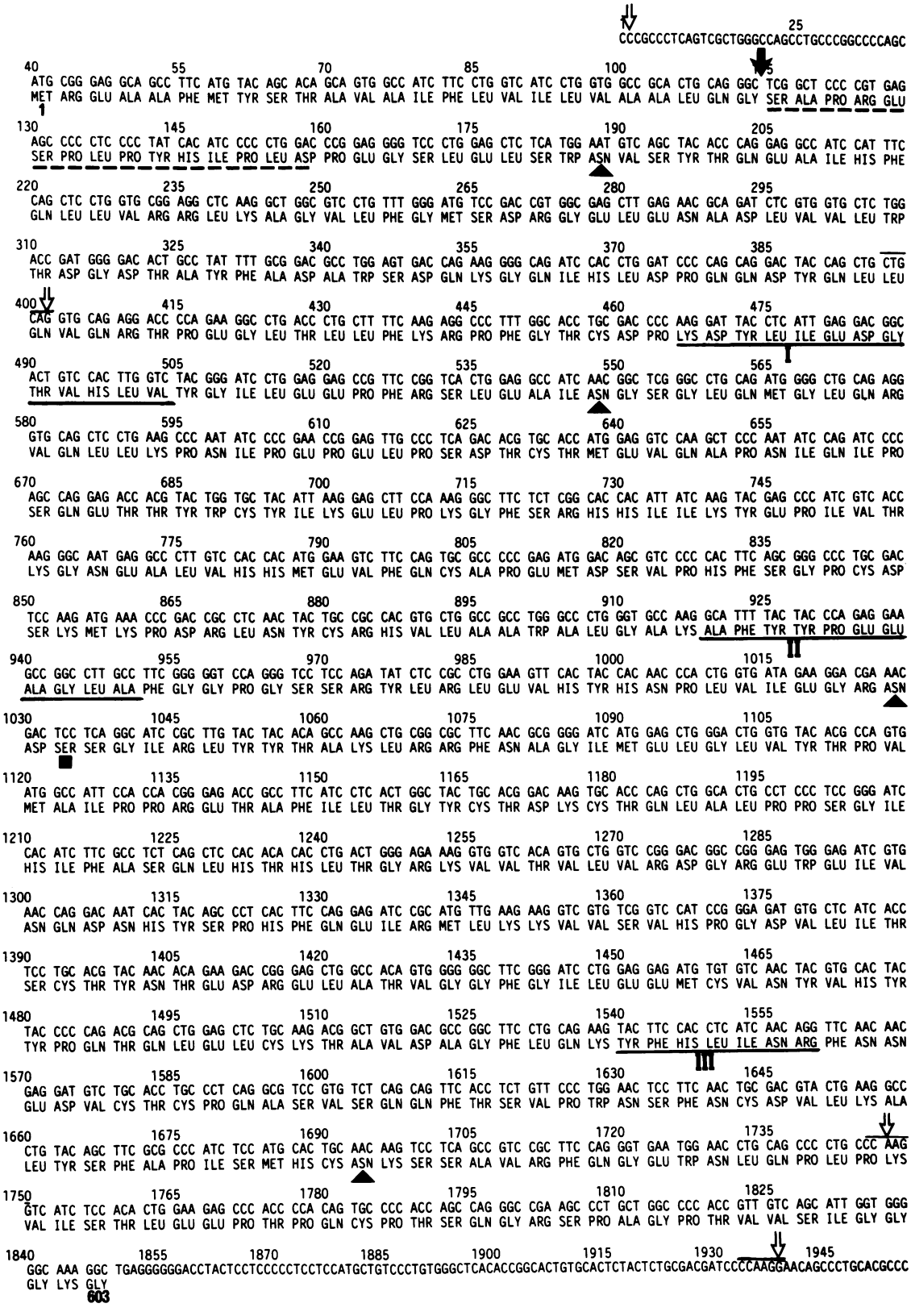

Fig. 2. Nucleotide and predicted amino acid sequences of human DBH as deduced from DBH2 clone. Nucleotides are numbered in the 5′ to the 3′ direction starting with the first residue following the *Eco*RI cloning site. The N-terminal methionine and the C-terminal glycine are numbered 1 and 603 respectively. The filled arrow indicates the putative cleavage site of the signal peptide. The broken line underlines the residues compared to the determined N-terminal sequence of bovine DBH. The roman numbers designate the stretch of amino acids that correspond to the three tryptic peptides. The Asn residues marked by filled triangles are potential N-glycosylation sites. The filled square shows a potential phosphorylation site. The open arrows delimitate the regions covered by the probes used for the S1 mapping analysis.

1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	
			ALA	GLU	SER	PRO	PHE	PRO	PHE	HIS	ILE	PRO	LEU	ASP	(a)
SER	ALA	PRO	ALA	GLU	SER	PRO	PHE	PRO	PHE	HIS	ILE	PRO	LEU	ASP	(b)
SER	ALA	PRO	ARG	GLU	SER	PRO	LEU	PRO	TYR	HIS	ILE	PRO	LEU	ASP	(c)

Fig. 3. Comparison of DBH N-terminal sequences. Lines a and b correspond to the two N-terminal sequences of bovine soluble DBH determined by Skotland *et al.* (1977), modified by Joh and Hwang (1986). Line c presents the deduced amino acid sequence of DBH2 clone, from residue 26 to residue 40.

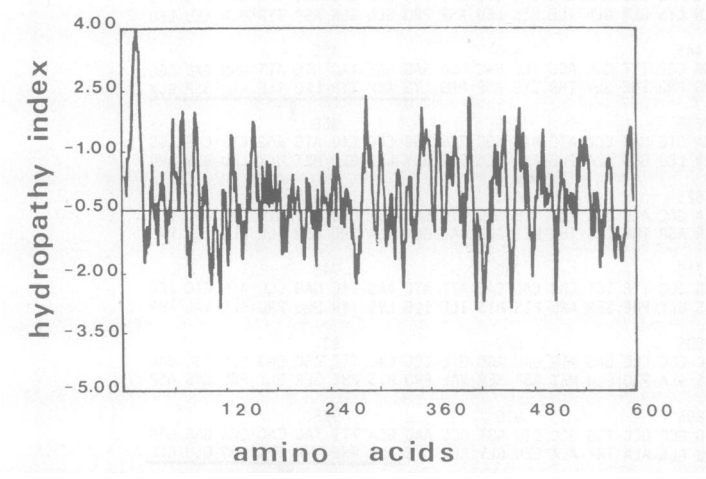

Fig. 4. Hydropathy profile of the predicted amino acid sequence of human DBH. Hydropathy values for a span of seven amino acid residues were calculated according to Kyte and Doolittle (1982).

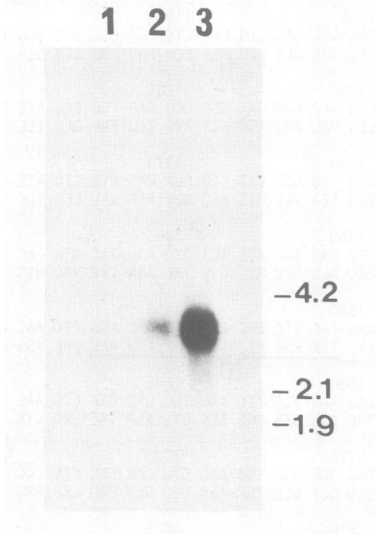

Fig. 5. Northern blot analysis of human DBH mRNA. Poly(A) RNA from human liver (**lane 1**, 3 μg mRNA), human adrenal medulla (**lane 2**, 1 μg mRNA) and human pheochromocytoma (**lane 3**, 1 μg mRNA) were hybridized with the 2.8-kb insert of clone DBH3 labelled by nick-translation. The size of DBH mRNA was calibrated with *Hin*dIII-digested λ DNA.

Predictions can be made concerning the sites of particular post-translational modifications occurring in the mature protein. DBH contains a high percentage of carbohydrates as a result of *N*-glycosylation, on average six per tetrameric molecule (Margolis *et al.*, 1984); this requires at least two putative glycosylation sites per monomer. In fact four potential sites (Hubbard and Ivatt,

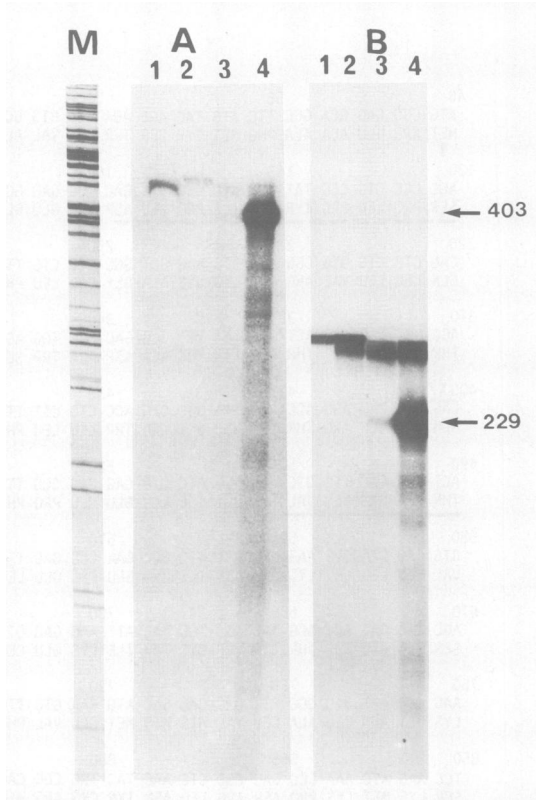

Fig. 6. Nuclease S1 analysis of the 5′ and 3′ ends of DBH RNA. The analysis was carried out with probes complementary to the 5′ end of clone DBH2 (**A**) and the 3′ end of the coding region (**B**). The limits of each probe are noted in Figure 2. The lengths of S1-protected fragments are indicated; **lane M** contains marker DNA fragments. **Lanes 1**, undigested probes; **lanes 2**, 10 μg yeast transfer RNA; **lanes 3**, 30 μg human adrenal medulla total RNA; **lanes 4**, 30 μg human pheochromocytoma total RNA. The exposure time was 3 h.

1981) are found, indicating that glycosylation is limited by the availability of some of these sites to the glycosylating enzymes. A potential phosphorylation site for calmodulin-dependent kinase is also present (Pearson *et al.*, 1985); interestingly, it overlaps with one of the possible glycosylation sites, a situation that might explain conflicting reports on the phosphorylation of mature DBH (Burgoyne and Geisow, 1982; Sabban *et al.*, 1983; McHugh *et al.*, 1985).

A comparison of the DBH sequence with the protein sequence data libraries of the National Biochemical Research Foundation (NBRF) and the EMBL showed no significant homologies. A more detailed comparison was done with bovine chromogranin A (Benedum *et al.*, 1986), human secretogranin I (Benedum *et al.*, 1987), rat and human TH (Grima *et al.*, 1985, 1987) and bovine PNMT (Baetge *et al.*, 1986) by using the statistical method of Smith *et al.* (1985) and Arratia *et al.* (1986). No significant homology between DBH and any of these proteins was found.

The hydrophobicity profile of the protein was established according to the procedure of Kyte and Doolittle (1982) and is presented in Figure 4. It reveals only one significant hydrophobic domain (amino acids 1−26) corresponding to the N-terminal signal peptide.

RNA analysis

Northern blot analysis of human pheochromocytoma and adrenal medulla mRNAs reveals a single species of ~ 3 kb (Figure 5). It has been suggested that membrane-bound and soluble DBH differ by a hydrophobic tail; such a situation could result from the occurrence of two different mRNAs arising by an alternative

splicing process. To test this hypothesis, we analysed DBH RNA by the S1 mapping technique, with a *Pst*I−*Eco*RI fragment corresponding to the 5′ end of the DBH2 clone and a *Sty*I fragment located in the 3′ end of the coding region (the limits of both probes are indicated in Figure 2). The result of such an experiment is presented in Figure 6. A single protected fragment, corresponding to the length of the probe minus the M13mp 18 primer and polylinker, is detected in each case, both with human pheochromocytoma and adrenal medulla RNAs. This result clearly establishes that no alternative splicing occurs in the regions covered by these probes.

Discussion

We present here the complete amino acid sequence of human DBH, deduced from a full-length cDNA clone, isolated by taking advantage of the high expression level of DBH in pheochromocytoma tumours.

The identity of the DBH clone, initially selected by immunoscreening a λgt11 expression library, was confirmed by two approaches. First, antibodies selected with fusion proteins generated by positive clones were shown to precipitate and titrate DBH activity. Second, the sequence of three internal peptides, obtained from purified human DBH, was found to be included in the deduced DBH sequence displayed in Figure 2. Furthermore, the N-terminal amino acid sequence of bovine soluble DBH reported by two groups (Skotland *et al.*, 1977; Joh and Hwang, 1986) exhibits a nearly complete homology with the deduced sequence between amino acids 26 and 40 (Figure 3). Only three differences, including two conservative substitutions, were found in the 15 determined residues, a result that underlines the relatedness of bovine and human DBH. It should be noted that Skotland *et al.* (1977) have reported for the soluble form of DBH two N-termini differing by three amino acids (Figure 3). The S1 mapping analysis of the 5′ extremity demonstrates that this peptide heterogeneity is not reflected in the mRNA species. This comparison has allowed us to identify Ser-26 as the N-terminal amino acid of the mature protein and to infer that the DBH translation product contains a 25-residue signal peptide, a deduction supported by the highly hydrophobic nature of this region (Figure 4). The existence of a signal peptide accounts for the exclusive location of DBH mRNA on membrane-bound polysomes (Sabban *et al.*, 1983).

Analysis of the predicted DBH sequence, in conjunction with S1 mapping experiments, has implications for the structure and the biosynthesis of the membrane-bound and soluble forms of the enzyme. Both species have been shown to be similar in their immunoreactivities (Slater *et al.*, 1981), binding affinities for various substrates (Brodde *et al.*, 1976; Aunis *et al.*, 1977), amino acid composition (Slater *et al.*, 1981; Sokoloff *et al.*, 1985) and peptide maps (Sokoloff *et al.*, 1985). The last two observations provide strong indication that the two forms are derived from a single gene. Moreover, the precursor−product relationship between the membrane-bound and soluble forms of DBH, established by Sabban *et al.* (1983, 1987) implies the existence of a single mRNA for the two forms of the protein. This prediction is confirmed by the results of the S1 mapping analysis of the 5′ and 3′ extremities of the coding region and by the *in vitro* translation experiments that yield a single band with both rat PC12 mRNA and bovine adrenal medulla mRNA (Sabban *et al.*, 1983; Joh and Hwang, 1986), as well as with human pheochromocytoma and adrenal medulla mRNAs (Benlot *et al.*, 1985 and this study, result not shown). The mol. wt of 67 527 daltons calculated from the deduced sequence of the DBH2 clone is compatible with that of the *in vitro* translation product; this value corresponds to the unprocessed protein with its signal peptide.

The differences in apparent mol. wt and amphiphilicity demonstrated by SDS−PAGE (Saxena and Fleming, 1983) and charge shift electrophoresis (Bjerrum *et al.*, 1979; Saxena and Fleming, 1983) between the membrane-bound and soluble forms of DBH suggest the presence of a hydrophobic moiety attaching the enzyme to the vesicular membrane. Inspection of the amino acid sequence depicted in Figure 4 reveals no obvious hydrophobic segment other than the N-terminal signal sequence. A few instances in which a protein is anchored to the membrane by an uncleaved signal peptide have been described (Van Rompuy *et al.*, 1982; Matsuda *et al.*, 1983). However, the homology existing between the cleavage site of secretogranin I (Benedum *et al.*, 1987) and that postulated for DBH, supports the contention that the DBH signal peptide is removed. Therefore, membrane attachment is likely to be due to a post-translational modification. This hypothesis accounts for the similarity in mol. wts of the *in vitro* translation product and of the membranous form of DBH, found in cells treated with tunicamycin, an inhibitor of glycosylation (Sabban *et al.*, 1983); the differences in mol. wt introduced by the cleavage of the signal peptide would be compensated, in the membrane-bound form, by the addition of the group responsible for membrane attachment.

Post-translational mechanisms, involving direct acylation or indirect acylation via phosphatidylinositol, have been invoked for a growing number of eukaryotic proteins to explain their interaction with the membrane (for reviews, see Cross, 1987; Sefton and Buss, 1987). Direct acylation can take the form of palmitylation or myristylation. In the case of DBH, palmitylation is unlikely according to the results of McHugh *et al.* (1985), and myristylation appears to require an N-terminal glycine, not present in the DBH sequence. Besides, proteins subjected to direct acylation are mostly unglycosylated (Olson and Spizz, 1986). In contrast, presently available information does not rule out glycosylphosphatidylinositolation (glypiation) as the mode of DBH membrane attachment. This process has been shown to concern a number of cell surface glycoproteins; it occurs immediately after translation, in line with the observation that the membrane-bound form of DBH is a precursor of the soluble form and it is not affected by tunicamycin (Ferguson *et al.*, 1986). According to this hypothesis, the soluble form of the protein would arise through a further post-translational event leading to the cleavage of the phospholipid moiety, and possibly a part of the peptide chain. A definitive answer to the mode of attachment of DBH to the membrane awaits further studies, such as the determination of the C-terminal sequence of the soluble form and analysis of the sensitivity of the membranous form to specific phospholipases, as well as transfection experiments with the DBH2 clone.

To detect signals that might be responsible for the targeting of DBH to the chromaffin vesicles, the sequence of the protein was compared to that of chromogranin A and secretogranin I. These two proteins were shown to have discrete regions of similarity at their NH_2 and COOH extremities (Benedum *et al.*, 1987). No significant similarity was detected between DBH and either of these proteins. However, it cannot be excluded that a functional signal remains undetected by the available programs. Alternately, the signal responsible for the intracellular transport to the chromaffin vesicle might not rely upon the primary structure of the proteins.

Comparison of the DBH amino acid sequence with either that of rat or human TH (Grima *et al.*, 1985, 1987) and bovine PNMT (Baetge *et al.*, 1986) reveals no significant homologies between

these proteins. This analysis conflicts with the report of Baetge *et al.* (1986) describing some similarity between TH and PNMT. This discrepancy is due to the less stringent method of sequence alignment adopted by these authors; the homology which is reported involves too many gaps to be significant. This point contrasts with the significant homology that exists between TH, phenylalanine hydroxylase (PH) and tryptophan hydroxylase (TPH), the three pterin-dependent aromatic amino acid hydroxylases, reflecting the functional similarity of these enzymes as well as their evolutionary filiation (Darmon *et al.*, 1986; Grenett *et al.*, 1987).

The DBH probe should prove to be fruitful for identifying and analysing diseases that may result from a defect in the expression or the structure of the enzyme. For instance, several cases of congenital deficiency in DBH activity have been reported (Man in'T Veld *et al.*, 1987). More importantly, catecholamines and serotonin are thought to play an important role in affective disorders. In this context, the study of an Amish pedigree revealed that the defect responsible for manic-depression, is located in the vicinity of the TH locus (Egeland *et al.*, 1987) suggesting that mutations in this gene may be important in the aetiology of this disease. Similarly, the DBH gene stands as a good candidate for investigation in correlation with psychiatric disorders.

Materials and methods

Anti-DBH serum

Anti-DBH serum was raised in rabbit by injection of pure human DBH as described by Antreassian *et al.* (1986).

Construction and screening of a λgt11 human pheochromocytoma library

RNA was prepared according to Lomedico and Saunders (1976) from a human pheochromocytoma tumour freezed in liquid nitrogen 30 min after surgery. Polyadenylated RNA was used to synthesize double-strand cDNA (Gubler and Hoffman, 1983) which was inserted into the *Eco*RI site of the expression vector λgt11 (Watson and Jackson, 1985). Approximately 150 000 recombinant bacteriophages were obtained. Half of these were screened using the specific anti-human DBH serum at a dilution of 1:1000, as described by Huynh *et al.* (1985), except that the second antibody was conjugated to horseradish peroxidase.

Purification of fusion proteins

Lysogenic clones of λgt11 DBH were constructed using the *Escherichia coli* strain Y1089. Sonicated freeze-thawed bacterial extracts obtained from induced cultures (Huynh *et al.*, 1985) were passed through a *p*-amino-β-thiogalactoside–Sepharose affinity column, as described by Ullmann (1984). The eluted fractions were immediately neutralizd by HCl to obtain a final pH of 7.

Specific antibody purification

About 200 μg of proteins were subjected to Western blotting (Towbin *et al.*, 1979). The specific bands were cut out, fragmented and washed for 1 h at room temperature in 50 ml phosphate-buffered saline (PBS) containing 20% calf serum and 0.1% Triton X-100. They were then incubated overnight at 4°C with 50 μl of anti-DBH serum diluted in 5 ml of the above solution, washed five times for 15 min at room temperature in 10 ml PBS containing 20% calf serum and 0.1% Triton X-100, and twice for 15 min in PBS alone. Specific immunoglobulins were eluted from the fragments at 4°C with 700 μl 100 mM glycine–HCl, pH 2.3 for 3 min. The eluate was immediately neutralized wtih 90 μl 1 M Tris-base and 30 μl of 1 M HCl were added to obtain a final pH of 7.5. Bovine serum albumin (BSA) was then added to a final concentration of 0.5 mg/ml. The elution process was repeated once again. The neutralized antibody solutions were pooled and applied to a Centricon column (Amicon) to be dialysed against PBS and concentrated three times to a final volume of 500 μl.

Inhibition of DBH activity by affinity-purified antibodies

Purified DBH (1.5 U) or pheochromocytoma homogenate (30 μg) were incubated with various amounts of selected specific antibodies, for 1 h at room temperature and 12 h at 4°C. When specified, protein A–Sepharose (Pharmacia) (15 mg), swollen in 10 mM sodium phosphate buffer, pH 7, was then added to the reaction mixture and incubated for 1 h at 4°C under agitation. The mixture was then centrifuged and the supernatant assayed for DBH activity.

DBH assay

DBH activity was assayed according to the spectrophotometric method of Wallace *et al.* (1973), except that a butanol extraction step was added before measuring the absorbance at 335 nm. One unit of DBH activity corresponds to one nanomole of octopamine produced/min at 37°C.

Sequencing of tryptic peptides of human DBH

DBH enzyme was purified from human pheochromocytoma as described by Antreassian *et al.* (1986). After SDS–PAGE of 0.8 mg of the preparation, the 74 000-dalton band was excised, electroeluted and lyophilized. The residue was washed in 1 ml 100% methanol, at room temperature and centrifuged for 5 min in a Microfuge. The pellet was resuspended in 90 μl of a solution containing 50 mM ammonium bicarbonate, 0.5 mM $CaCl_2$ and 5 μg TPCK-trypsin (Worthington), incubated for 15 h at 37°C and subjected to HPLC on a LKB system equipped with a Vydac RP18 column using an acetonitrile gradient in TFA as described by Benedum *et al.* (1986). Individual peaks were collected, concentrated in a Speedvac and sequenced in a gas phase sequanator as described by Franck and Trosin (1985).

DNA sequencing

The various inserts (*Eco*RI fragments) from λgt11 DBH clones were mapped with several restriction enzymes. Fragments to be used for sequencing were isolated and subcloned into M13mp8 or M13mp18 vectors. The M13 subclones obtained were subjected to DNA sequence analysis according to the method of Sanger *et al.* (1977).

Northern blots

Polyadenylated RNA from human pheochromocytoma tumour, liver and adrenal medulla were analysed by Northern blotting according to Faucon Biguet *et al.* (1986).

S1 mapping analysis

Single-stranded recombinant M13 DNA (1 μg) was annealed with M13 sequencing primer (P.L. Biochemicals) and the labelled complementary strand was synthesized with Klenow polymerase. The double-stranded DNAs were digested with *Eco*RI and the labelled single-stranded inserts were purified by electrophoresis on a 5% denaturing acrylamide gel. The probes (100 000 c.p.m.) were hybridized to total RNA in 80% formamide at 45°C for 15 h after which they were digested with S1 nuclease (400 U/ml, Boehringer) at 37°C for 1 h. The samples were then extracted with phenol, precipitated and analysed on a 6% denaturing acrylamide gel.

Acknowledgements

We are grateful to P.A.Baeuerle and W.B.Huttner for help in performing amino acid digestion and HPLC analysis in their laboratory. We thank A.Henaut and C.Mugnier for their assistance in computer sequence analysis; J.F.Julien, A.Menez, E.Petrochilo, P.Vernier, J.L.Popot and A.Rousselet for helpful discussions; Dr X.Bertania, Hopital Cochin, Paris, Dr P.F.Ploin, Hopital Broussais, Paris, Dr R.Mornex, Hopital Antiquaille, Lyon, for kindly providing human pheochromocytoma; C.Herbert for critical reading of the manuscript. G.Peudevin and D.Samolyk for efficient technical assistance. This work was supported by grants from the Centre National de la Recherche Scientifique, the Institut National de la Santé et de la Recherche Médicale, the Fondation pour la Recherche Médicale Française, and Rhône-Poulenc Santé.

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Received on September 16, 1987

Note added in proof

These sequence data have been submitted to the EMBL/GenBank data libraries under the accession number Y00096.