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Complete nucleotide and deduced amino acid sequence of bovine phenylethanolamine *N*-methyltransferase: Partial amino acid homology with rat tyrosine hydroxylase

(catecholamine enzymes/epinephrine biosynthesis/metallothionein vector/cDNA sequence/sequence homology)

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ABSTRACT **We report here the isolation of a cDNA clone containing the full coding region of bovine phenylethanolamine *N*-methyltransferase (PNMTase, EC 2.1.1.28, *S*-adenosyl-L-methionine:phenylethanolamine *N*-methyltransferase). The complete nucleotide sequence of the cDNA has been determined, and the amino acid sequence of PNMTase deduced. Cultured cells transfected with an expression vector containing this cDNA produced high levels of PNMTase enzymatic activity. Antibodies specific for tyrosine hydroxylase [EC 1.14.16.2, tyrosine 3-monooxygenase; L-tyrosine, tetrahydrobiopterine: oxygen oxidoreductase (3-hydroxylating)], the first enzyme in the catecholamine pathway, possess a striking affinity for the PNMTase protein synthesized *in vitro*. Comparison of the deduced amino acid sequence of bovine PNMTase to rat tyrosine hydroxylase reveals that PNMTase shares significant homology with tyrosine hydroxylase and supports previous protein and immunological data suggesting that the catecholamine biosynthetic enzymes are structurally related.**

The catecholamine biosynthetic pathway is composed of four enzymes responsible for the enzymatic conversion of the amino acid tyrosine to the three principal catecholamine compounds dopamine, norepinephrine, and epinephrine (1). Phenylethanolamine *N*-methyltransferase (PNMTase; EC 2.1.1.28; *S*-adenosyl-L-methionine:phenylethanolamine *N*-methyltransferase) is the terminal enzyme in the catecholamine pathway, catalyzing the synthesis of epinephrine from norepinephrine (2). The enzyme is present in high levels in the chromaffin cells of the adrenal medulla, where epinephrine serves as a hormone (3). Sympathetic ganglia contain a minor population of small intensely fluorescent cells (SIF cells), which contain PNMTase catalytic activity and synthesize ephinephrine (4–6). This is reminiscent of the adrenal medulla in that two catecholaminergic cell types exist within a specific population of cells producing norephinephrine and epinephrine as their final transmitter products. PNMTase is also found in neuronal perikarya of the medulla oblongata, hypothalamus, and retina where epinephrine is presumed to function as a neurotransmitter (7–13).

We have generated molecular probes to the catecholamine enzymes as a means to further investigate their expression at the mRNA level as well as to characterize the genes coding for these proteins. It has been suggested that the catecholamine enzymes are related to one another not only as sequential enzymes in a biosynthetic pathway but also as proteins sharing immunological and structural similarities (14–16).

To obtain information about the structure of the PNMTase molecule and to further define the extent of the evolutionary relationships among these enzymes, a full-length cDNA clone to PNMTase mRNA was isolated, and the entire nucleotide and amino acid sequences were determined. We show that this PNMTase cDNA is capable of programming the *in vitro* synthesis of PNMTase that is both enzymatically active and extremely cross reactive with antisera against tyrosine hydroxylase [TyrHase; EC 1.14.16.2.; tyrosine 3-monooxygenase; L-tyrosine tetrahydrobiopterine: oxygen oxidoreductase (3-hydroxylating)]. These antigenic similarities are further revealed by comparison of the deduced amino acid sequences of TyrHase and PNMTase.

MATERIALS AND METHODS

Isolation of PNMTase cDNA Clone. Polysomal poly(A)$^+$ mRNA was prepared from bovine adrenal medulla by a modification of the procedure of Holbrook and Brown (17) as described (18). A λgt11 expression library was constructed as described in detail elsewhere (19). Approximately 120,000 phage plaques were transferred to nitrocellulose and screened by hybridization with a ^{32}P-labeled PNMTase-22 cDNA (20). Plaque-purified clones were restriction mapped and sequenced by the chemical modification method of Maxam and Gilbert (21). Detailed methods for nick-translation, library construction, and screening are described in Maniatis *et al.* (22).

Metallothionein PNMTase Expression Vector and Cell Transfection. PNMTase-17 cDNA was ligated in both orientations behind the mouse metallothionein (MT)-I promoter (23–37), see Fig. 2*A*. The plasmid expression vector, pNUT (B. Davison, personal communication), also contains a cDNA encoding the mutant form of dihydrofolate reductase (28), driven by the simian virus 40 promoter. The MT-PNMTase construct was transfected into the baby hamster kidney cell line (BHK) (29), using standard $CaPO_4$ precipitation procedures (26, 30). Cell lines, which had stably incorporated the plasmid, were selected by including methotrexate to a final concentration of 1 mM in the culture media.

Transcription at the heavy metal responsive MT-I promoter was induced by adding $ZnSO_4$ to the culture media at a final concentration of 100 μM (26). Cells were harvested after a 16-hr incubation, and the cell pellets were sonicated in 200 μl of homogenization buffer (10 mM potassium phosphate, pH 7.0/0.2% Triton X-100) and centrifuged for 10 min in an Eppendorf centrifuge. Supernatants were immediately frozen at −70°C or assayed directly for PNMTase enzyme activity (18).

***In Vitro* Translation and Transcription.** Polysomal poly(A)$^+$ mRNA isolated as described above was translated in the

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Abbreviations: PNMTase, phenylethanolamine *N*-methyltransferase; TyrHase, tyrosine hydroxylase; MT, metallothionein; bp, base pair(s); tTyrHase, trypsin-treated tyrosine hydroxylase.

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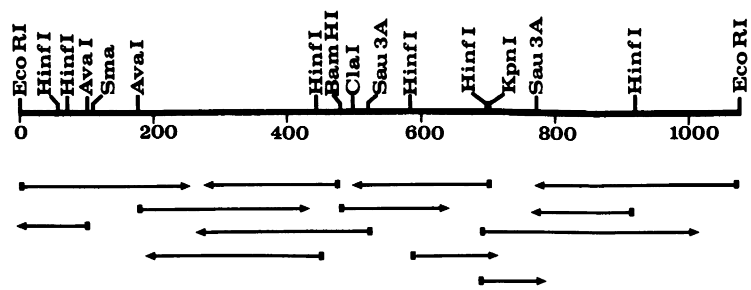
reticulocyte translation system of Pelham and Jackson (31). Translation reactions contained 0.16 mM $Mg(OAc)_2$, 60 mM KOAc, poly(A)$^+$ mRNA at 15 μg/ml, lysate, [^{35}S]methionine, and cocktail according to the supplier's (New England Nuclear) recommendations. Translation reactions were incubated at 30°C for 70 min. Antibodies to TyrHase (32) and PNMTase (18) were added to 25-μl aliquots and incubated for 16 hr at 4°C. Immunoprecipitates were isolated with protein A-Sepharose CL-4B (Pharmacia), according to Dobberstein *et al.* (33). Washed immunoprecipitates were boiled in $NaDodSO_4$ sample buffer and analyzed by $NaDodSO_4$/polyacrylamide gel electrophoresis (34). Gels were fixed and impregnated with New England Nuclear Enhance, dried, and exposed to Kodak XAR x-ray film.

SP6 plasmid DNA containing the 1.06-kilobase PNMTase cDNA insert was linearized by digestion with *Sac* I and used as a template for the synthesis of PNMTase mRNA by SP6 polymerase. The transcription reaction contained 0.5 mM concentrations of m^{7}G(5')ppp(5')G and all four ribonucleoside triphosphates, 20 units of SP6 polymerase using the conditions described by Green *et al.* (35). Transcribed mRNA was digested with RNase-free DNase (Bethesda Research Laboratories) at 20 units/μl, in the presence of 2 units of RNasin (Promega Biotec, Madison, WI) at 37°C for 15 min. Following extraction with phenol/chloroform, the mRNA was purified from unincorporated nucleotides by spin column chromatography on Sephadex G-50 (22). The purified mRNA was translated at a final concentration of 100 μg/ml, using the conditions described above. Complete translation reactions were incubated with specific antisera, fractionated by $NaDodSO_4$/PAGE, and analyzed by fluorography.

RESULTS AND DISCUSSION

cDNA Cloning and DNA Sequencing. A cDNA expression library containing approximately 107 recombinants was constructed in λgt11 (36) using poly(A)$^+$ mRNA isolated from bovine adrenal medulla. A 1.06-kilobase cDNA clone,

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          -40        -30        -20        -10        -1  1
           CGTGATGTCCTCAAAGTGGGCACAGGCGCTGAGCAGCAGC ATG AGC GGG ACA GAC CGG AGT
                                                    MET Ser Gly Thr Asp Arg Ser
         10                                      20
CAG GCG GCG GGC GCA GTG CCC GAC TCA GAC CCG GGC CTG GCG GCG GTC TCC TCG GCC TAC
Gln Ala Ala Gly Ala Val Pro Asp Ser Asp Pro Gly Leu Ala Ala Val Ser Ser Ala Tyr
         30                                      40
CAG CGC TTT GAG CCC CGC GCC TAC CTC CGC AAC AAC TAC GCG CCC CCG AGG GGG GAC CTG
Gln Arg Phe Glu Pro Arg Ala Tyr Leu Arg Asn Asn Tyr Ala Pro Pro Arg Gly Asp Leu
         50                                      60
AGC TGC CCC GAC GGC GTC GGG CCT TGG AAG CTG CGC TGC TTG GCT CAG ACC TTC GCC ACC
Ser Cys Pro Asp Gly Val Gly Pro Trp Lys Leu Arg Cys Leu Ala Gln Thr Phe Ala Thr
         70                                      80
GGT GAG GTG TCT GGC CGC ACC CTC ATT GAC ATC GGT TCA GGA CCC ACT ATA TAC CAG CTG
Gly Glu Val Ser Gly Arg Thr Leu Ile Asp Ile Gly Ser Gly Pro Thr Ile Tyr Gln Leu
         90                                      100
CTC AGC GCC TGT GCC CAC TTT GAG GAC ATC ACC ATG ACA GAT TTC CTG GAG GTG AAC CGC
Leu Ser Ala Cys Ala His Phe Glu Asp Ile Thr MET Thr Asp Phe Leu Glu Val Asn Arg
         110                                     120
CAG GAG CTG AGG CTC TGG CTG CGA GAA GAG CCT GGG GCT TTC GAC TGG AGC GTG TAC AGC
Gln Glu Leu Arg Leu Trp Leu Arg Glu Glu Pro Gly Ala Phe Asp Trp Ser Val Tyr Ser
         130                                     140
CAG CAT GTC TGC CTC ATC GAG GGC AAG GGG GAA TCC TGG CAG GAG AAG GAG TGC CAG CTG
Gln His Val Cys Leu Ile Glu Gly Lys Gly Glu Ser Trp Gln Glu Lys Glu Cys Gln Leu
         150                                     160
CGA GCC AGG GTG AAG AGG ATC CTG CCC ATC GAT GTG CAC CGG CCC CAG CCC CTG GGT GCT
Arg Ala Arg Val Lys Arg Ile Leu Pro Ile Asp Val His Arg Pro Gln Pro Leu Gly Ala
         170                                     180
GGA GGC CTG GCA CCC CTG CCT GCC GAC GCC CTG GTC TCT GCC TTC TGC CTG GAG GCT GTG
Gly Gly Leu Ala Pro Leu Pro Ala Asp Ala Leu Val Ser Ala Phe Cys Leu Glu Ala Val
         190                                     200
AGT CCA GAC CTG GCC AGC TTC CAG CGG GCC CTG GAC CAC ATC ACC ACA CTG CTG AGG CCT
Ser Pro Asp Leu Ala Ser Phe Gln Arg Ala Leu Asp His Ile Thr Thr Leu Leu Arg Pro
         210                                     220
GGG GGG CAC CTC CTC CTC ATC GGA GCC CTG GAG GAG TCA TGG TAC CTG GCT GGG GAG GCC
Gly Gly His Leu Leu Leu Ile Gly Ala Leu Glu Glu Ser Trp Tyr Leu Ala Gly Glu Ala
         230                                     240
AGG CTG GCG GTG GTG CCC GTG CGC GAG GAG GAG GTG AGG GAG GCC CTG GTG CGG AGC GCT
Arg Leu Ala Val Val Pro Val Arg Glu Glu Glu Val Arg Glu Ala Leu Val Arg Ser Ala
         250                                     260
ATG AGG TGC GGG ATC TGC GCA CTA CAC CTA CAC CAT GCC TGC CCA CCT TCA GAC AGG TGT
MET Arg Cys Gly Ile Cys Ala Leu His Leu His His Ala Cys Pro Pro Ser Asp Arg Cys
         270                                     280             284
AGA CGA TGT CAA GGG CAT CTT CTT CAC CTG GCC CAG AAG AAG GTG GGG GTG TGA GGCCCC
Arg Arg Cys Gln Gly His Leu Leu His Leu Ala Gln Lys Lys Val Gly Val Ter

CGTGCACGCAGGGCCAATGGCCCTCACCCGCCTAGACTCCCTGTTGTCCGAGGTGGCCTCAATAAAGGAATAAGTCCG

TCAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAA

AAAA
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FIG. 1. Nucleotide sequence and deduced amino acid sequence of cDNA clone encoding bovine adrenal medullary PNMTase. (*Upper*) Diagram of the restriction map and strategy used for sequencing the bovine cDNA clone PNMTase-17. (*Lower*) Complete nucleotide sequence of PNMTase-17. Initiator methionine is underlined at position 1. An open reading frame extends from nucleotide 40 to 882 where translation is terminated at the underlined TGA stop codon. The poly(A) addition recognition sequence "AATAAA" is underlined and located 15 nucleotides on the 5' side of the poly(A) start site. The predicted amino acid sequence is depicted below the nucleotide sequence.

(PNMTase-17), was isolated from this library, by hybridization to a 350-base-pair (bp) partial length cDNA for bovine PNMTase (PNMTase-22) (20). The restriction map and the sequencing strategy are shown in Fig. 1, *Upper*. The complete nucleotide and predicted amino acid sequences are depicted directly below. PNMTase-17 cDNA contains 1062 bp, of which 852 bp represent the coding region. There are 40 bp of 5′-untranslated sequence, and 167 bp of 3′-untranslated sequence containing 80 nucleotides of poly(A) tail. The ATG start codon is preceded by the residues "GCAGC" at positions −6 to −1, respectively. This sequence closely resembles the "CCACC" nucleotide region characterizing the initial codon sequences of many eukaryotic mRNAs (37, 38). The sequence "AATAAA," accepted as the consensus signal sequence for poly(A) addition (39), is located 15 bp to the 5′ side of the start of the poly(A) tail. The entire coding sequence of bovine PNMTase is contained in a single open reading frame of 852 nucleotides encoding a protein of 284 amino acids. The predicted M_r of 31,115 agrees well with the

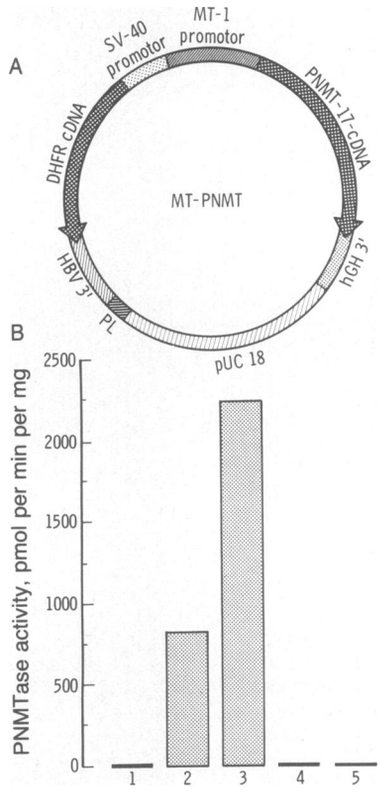

FIG. 2. MT-PNMTase expression vector. (*A*) PNMTase-17 cDNA was inserted behind the mouse MT-I promoter including the sequences from −650 to +7 (29). A 320-bp *Sma* I–*Nsi* I fragment containing the 3′ end of human growth hormone gene (hGH) [including the poly(A) addition sequence and other sequences important for maturation of mRNA 3′ ends] was joined to the 3′ end of PNMTase cDNA. The dihydrofolate reductase, cDNA is driven by the simian virus 40 promoter and has attached to its 3′ end 1200 bp of the 3′ end of the hepatitis B virus gene containing the necessary polyadenylylation maturation signals. The entire pUC-18 plasmid, including the polylinker (PL), separates the 3′ ends of the promoter driven cDNAs. (*B*) PNMTase enzymatic activity expressed in BHK cells. (Bar 1) BHK cells; 0 pmol per min per mg. (Bar 2) BHK cells carrying the stable integrant of MT-PNMTase as shown above; 778 pmol per min per mg. (Bar 3) BHK cells induced with 100 μm $ZnCl_2$ for 16 hr carrying the MT-PNMTase construct described above; 2250 pmol per min per mg. (Bar 4) BHK cells carrying the MT-PNMTase construct with PNMTase cDNA in the opposite orientation; 0 pmol per min per mg. (Bar 5) BHK cells induced with 100 μm $ZnCl_2$ for 16 hr carrying the MT-PNMTase construct with the PNMTase cDNA in the opposite orientation; 0 pmol per min per mg.

M_r 31,000 determined by $NaDodSO_4$ gel electrophoresis of the purified enzyme.

MT-PNMTase Expression. As shown in Fig. 2*A*, the PNMTase-17 cDNA has been inserted downstream from the mouse MT-I promoter element in an expression vector containing the selectable marker gene dihydrofolate reductase. Following transfection into BHK cells and selection in methotrexate containing media, transformed cell lines were isolated containing stable integrants of the MT-PNMTase construct. Extracts made from these cell lines were assayed for the ability to *N*-methylate phenylethanolamine using the cofactor *S*-adenosyl-L-[^{3}H]methionine in a PNMTase enzyme assay. As shown in Fig. 2*B*, bar 1, BHK cells by themselves contain absolutely no PNMTase activity, whereas BHK cells, which have stably incorporated the MT-PNMTase construct, express 778 pmol per min per mg of PNMTase enzyme activity (bar 2). As shown by bar 3, transcriptional induction of the MT promoter with $ZnCl_2$ increases PNMTase enzyme activity almost 3-fold to 2215 pmol per min per mg, as compared to uninduced levels. Conversely, when the PNMTase-17 cDNA is inserted into the expression vector in the opposite orientation, the selected lines express no PNMTase enzyme activity as shown by bars 4 and 5. These results clearly establish the identity of this clone and strongly indicate that this cDNA contains the entire protein coding domain of PNMTase.

***In Vitro* Transcription and Translation.** We (15), and others (16), have demonstrated that antibodies specific for a single, purified catecholamine biosynthetic enzyme appear to recognize more than one catecholamine enzyme from *in vitro* translated protein products. The most unusual example of this cross reactivity was the ability of the antibody to the trypsin-digested form of TyrHase (tTyrHase) (32) to precipitate primarily PNMTase from *in vitro* translation products.

As shown in Fig. 3*A*, lane 1, antibody to rat PNMTase precipitates a M_r 31,000 protein, identical in size to that found *in vivo* (18). When specific antisera raised against tTyrHase is incubated with bovine adrenal mRNA translation products, a similar M_r 31,000 protein is precipitated (Fig. 3*A*, lane 3). Although immunoblot analysis, enzymatic inhibition studies, and immunocytochemical localizations have confirmed that this antiserum is absolutely specific for purified TyrHase (32, 40–43), it is apparent that PNMTase synthesized *in vitro* shares some antigenic determinants with the tTyrHase which are not present or exposed on the native PNMTase molecule. Preadsorption of the translation reaction with excess PNMTase antibody results in the subsequent inability of

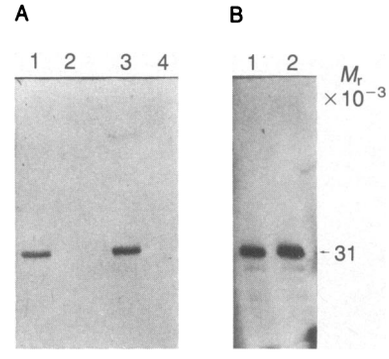

FIG. 3. ^{35}S-labeled proteins immunoprecipitated from *in vitro* translation products (*A*) of bovine adrenal medulla and (*B*) from *in vitro* transcription of PNMTase-17 cDNA. (*A*) Immunoprecipitated PNMTase protein: lane 1, PNMTase antibody; lane 2, tTyrHase antibody after preadsorption with PNMTase antibody. Immunoprecipitated tTyrHase protein: lane 3, tTyrHase antibody; lane 4, PNMTase antibody after preadsorption with tTyrHase antibody. (*B*) Antisera: lane 1, PNMTase antibody; lane 2, tTyrHase antibody.

tTyrHase antibody to precipitate the M_r 31,000 protein, indicating that this protein is indeed PNMTase (Fig. 3*A*, lane 2). Conversely, when excess tTyrHase antibody is used to preadsorb the translation mixture, the PNMTase antibody no longer precipitates PNMTase (Fig. 3*A*, lane 4). These results show that newly synthesized PNMTase is readily recognized by an antibody that is very specific for purified TyrHase. This occurs in spite of the correspondingly high level of translatable TyrHase mRNA present but does not occur when antibody raised against the holoenzyme of TyrHase (M_r 60,000 subunit) is used (15).

The availability of the full-length PNMTase-17 cDNA clone isolated here has allowed us to examine this cross reactivity in isolation, using an *in vitro* SP6 system coupling PNMTase-17 transcription and translation. The full-length PNMTase-17 cDNA insert was subcloned into an SP6 plasmid vector (43). RNA was transcribed from the linearized SP6-PNMTase-17 DNA template using SP6 polymerase as described (43), and the resultant transcripts were used to program an *in vitro* translation system. As shown in lanes 1 and 2 of Fig. 3*B*, both PNMTase and tTyrHase antibody recognize the sole protein product transcribed from PNMTase cDNA and translated *in vitro*. These results, as well as previous reports indicating that the catecholamine enzymes purified from bovine adrenal medulla possess common sized proteolytic peptides having similar amino acid compositions (14, 15), further support the contention that TyrHase and PNMTase, the first and fourth enzymes in the catecholamine pathway, share similarities in their primary structure.

Amino Acid Sequence Homology. To analyze more fully the extent of this similarity, we have compared the cDNA-derived amino acid sequence of bovine PNMTase to the deduced amino acid sequence of rat TyrHase (44), as shown in Fig. 4.

The most significant homologies become apparent when the entire PNMTase sequence is aligned either with the amino-terminal 218 amino acids or TyrHase (Fig. 4*A*), or with the carboxyl-terminal 264–454 amino acids of TyrHase (Fig. 4*B*). Comparison of the amino acid residues 8–44 of PNMTase with residues 10–46 in TyrHase results in 15 matches out of 42 positions (35.7%). Alignment of residues

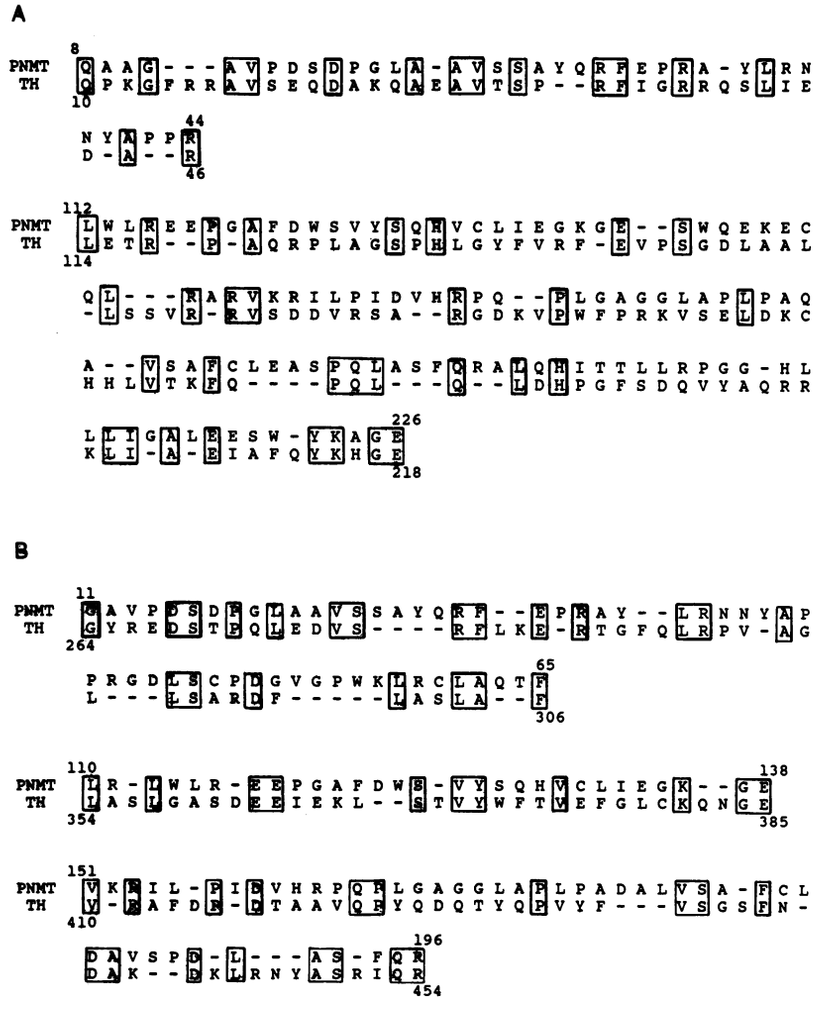

FIG. 4. Comparisons between bovine PNMTase and rat TyrHase-deduced amino acid sequences. (*A*) Optimal alignments of PNMTase to the amino-terminal portion of TyrHase. Identical residues are boxed; amino acid gaps (-) have been inserted to maximize the alignment. Comparison of PNMTase residues 8–44 with TyrHase residues 10–46. Positions compared, 42; gaps, 10; identities, 15; homology, 35.7%. Comparison of PNMTase residues 112–226 with TyrHase residues 114–218. Positions compared, 124; gaps, 30; identities, 31; homology, 25%. (*B*) Optimal alignments of PNMTase to the carboxyl-terminal portion of TyrHase. Comparison of PNMTase residues 11–65 with TyrHase residues 264–306. Positions compared, 59; gaps, 19; identities, 21; homology, 35.6%. Comparison of PNMTase residues 110–138 with TyrHase residues 354–385. Positions compared, 34; gaps, 7; identities, 11; homology, 32.3%. Comparison of PNMTase residues 151–196 with TyrHase residues 410–454. Positions compared, 53; gaps, 15; identities, 18; homology, 34%. Initial alignments were obtained by using sequence comparing program (Microgenie) developed by Beckman. Final alignments were adjusted by insertion of amino acid gaps to maximize homology, and these alignments were tested for significance by comparison to 100 jumbled sequences of identical amino acid composition and length using the "malign" program.

112–226 in PNMTase with residues 114–218 of TyrHase results in 31 identities out of 124 positions compared (25%). Alignment of PNMTase residues 11–65 with TyrHase residues 264–306, located in the carboxyl half of the molecule, resulted in 35.6% identities. Comparison of residues 110–138 in PNMTase with residues 354–385 of TyrHase leads to an identity of 32.3%, and comparison of PNMTase residues 151–196 with TyrHase residues 410–454 produces an overall homology of 34%. Comparison at the nucleotide level reveals that the DNA sequences have diverged more rapidly. Only 36% of the homologous amino acids have identical triplet codon sequences, whereas 64% show predominantly third-base degeneracy. These observations are consistent with the more rapid change of DNA sequences (as predicted by the degeneracy of the genetic code), during the evolution of two "related" proteins. This is especially true when comparing DNA sequences from two different species, as is presented here.

Sequence homologies have also been reported for sequential enzymes in ketoadipate biosynthesis, a multienzyme pathway for the metabolism of catechol and protocatechuate substrates in bacteria (45). Comparisons of the amino-terminal sequences of the enzymes that mediate sequential metabolic steps in this pathway reveals an overall homology of 30% (46). Enzymes catalyzing reactions separated by one or two metabolic steps in this pathway show regions of 21 and 38 residues exhibiting identities of 36% and 31%, respectively (47, 48). Furthermore, antisera raised against an individual enzyme in the pathway cross react with other enzymes in the same pathway (47).

In yet another *Escherichia coli* multienzyme pathway, that for the biosynthesis of threonine and methionine, the first and the third enzymes share interspersed sequence homologies analogous to those reported in our study. Specifically, regions of approximately 130 and 250 amino acid residues have been identified in these two enzymes that possess overall homologies of 20–50% (49).

Because PNMTase is an integral component in epinephrine biosynthesis, analysis of its structure can be expected to lend insights into the enzymatic and regulatory mechanisms involved in this process. It is clear, from the coupled *in vitro* transcription–translation experiments and the amino acid sequence comparisons presented above, that there exists a significant relationship between the primary structures of TyrHase and PNMTase. Comparison of the amino acid sequences of both enzymes in the same species should establish the true degree of homology between these two enzymes.

Future studies defining the three-dimensional structures of both proteins and establishing the relationship of these regions to their functional aspects may more clearly elucidate the mechanisms by which these enzymes evolved.

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