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A natural variant of bovine dopamine β-monooxygenase with phenylalanine as residue 208: purification and characterization of the variant homo- and heterotetramers of $(F208)_4$ and $(F208)_2(L208)_2$

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Abstract Bovine dopamine β-monooxygenase was purified from each of 18 individual adrenal glands by the method we have developed for the rapid purification of the enzyme from a single adrenal gland. Differential peptide mapping of the 18 enzyme preparations following fluorescence labeling of their cysteine residues revealed the presence of a novel variant with Phe as residue 208 in 14 adrenal glands; seven of them were homozygous for the variant allele and the remaining seven heterozygous. The variant enzyme was a tetramer and exhibited kinetic and structural properties similar to those of the wild-type tetramer $(L208)_4$. These results indicate an allelic polymorphism and co-dominant expression of the two alleles of the enzyme gene.

Key words: Dopamine β-monooxygenase; Variant; Polymorphism; Tetramer; Peptide mapping; Bovine

1. Introduction

Dopamine β-monooxygenase (EC 1.14.17.1, DβM) catalyzes the conversion of dopamine to noradrenaline within catecholamine secreting vesicles of adrenal medullary cells and adrenergic neurons (for reviews, see [1,2]). DβM also plays an essential role in fetal development through noradrenaline biosynthesis [3,4]. Bovine DβM is a tetramer consisting of two disulfide-linked dimers and exists as both soluble and membrane-bound forms [5–8]. Purified DβM samples often exhibit two or three protein bands as analyzed by SDS-PAGE [9]. Since DβM is encoded by a single-copy gene, the structural heterogeneity originates from posttranslational modifications such as limited proteolysis and glycosylation.

The complete primary structure of bovine DβM has been deduced from cDNAs cloned from bovine adrenal glands [10–12]. However, the reported sequences are not identical to each other. We use the sequence and residue numbering reported by Lewis et al. [11] as a standard. Then the following substitutions were found in the sequences based on cDNAs: R198C, E260R, T261D, I262H, A342R, Q443Y, R460E, or H581Q. Since each adrenal gland used to isolate cDNA was locally acquired, the differences strongly suggest the allelic polymorphism of the bovine DβM gene. In this context, it is interesting that kinetic and structural properties of DβM differ among preparations: Fleming et al. reported relatively low values of the equilibrium constant for the association of disulfide-linked dimers to tetramer (5.6×10^5–8.3×10^8 M^{-1}) at pH 5.0–5.5, and non-hyperbolic dependence of the initial velocity on tyramine concentration due to reversible enzyme oligomerization [13,14]. On the other hand, Klinman et al. observed no relationship between kinetic properties and enzyme concentration in the range of 0.1–1000 μg/ml [2,15]. All the DβM preparations purified by us showed a relatively stronger association of dimer to tetramer ([16]; unpublished results). These differences in enzyme properties may be due to differences in the primary structure of DβM.

The detailed tertiary and quaternary structures of DβM are not yet elucidated. However, many residues important for the enzyme activity have been determined: Y216, H249, H398, M473, Y477, and R503 [17–21]. When DβM is fully reduced and digested with trypsin, all these residues except for R503 are found in tryptic fragments containing one or two cysteine residues [16]. Thus, comparative mapping of cysteine-containing tryptic peptides of various DβM preparations should be effective for screening genetic variants of DβM with amino acid substitutions at positions near the residues essential for the activity.

In this study, we developed a method for rapid purification of DβM from a single adrenal gland, and obtained pure DβM (100–200 μg) from each of 18 bovine adrenal glands. We found that most of the DβM samples contained a novel tryptic fragment with the N-terminal sequence of APDV-FIPGQQTTYWCYV. We report kinetic and structural properties of the variant homo- and heterotetramers of $(F208)_4$ and $(F208)_2(L208)_2$ in comparison with those of the wild-type enzyme of $(L208)_4$.

2. Materials and methods

2.1. Materials

Adrenal glands from Japanese black cattle were obtained from a slaughterhouse in Kyoto. Tyramine hydrochloride, DTT, TPCK-trypsin, and Con A-Sepharose were purchased from Sigma; disodium fumarate, ascorbic acid, MES, and HEPES were from Nacalai Tesque; I-AEDANS was provided by Research Organics; and catalase was obtained as a crystalline suspension (65 000 units/mg) from Boehringer Mannheim. All other chemicals used were of analytical grade.

2.2. Enzyme purification from a single adrenal gland

The medulla was dissected from a fresh adrenal gland, and homogenized in three volumes of 8 mM potassium phosphate, pH 7.2, containing catalase (0.1 mg/ml) [22]. After 20 min incubation in ice-water under stirring, the homogenate was centrifuged at 16 000×g for

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Abbreviations: DβM, dopamine β-monooxygenase; DTT, dithiothreitol; *I*, ionic strength; I-AEDANS, *N*-iodoacetyl-*N'*-(5-sulfo-1-naphthyl)ethylenediamine; SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis

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10 min. The supernatant (crude extract) was subjected to polyethylene glycol fractionation according to Ljones et al. [23] in the presence of catalase (0.1 mg/ml). The enzyme solution was then applied to a DEAE-Toyopearl column (Tosoh, Tokyo; 10×150 mm) equilibrated with 20 mM potassium phosphate, pH 7.5. The flow rate was 1.0 ml/min. After 20 min washing with the same buffer, the enzyme was eluted with a linear gradient of 0–0.5 M NaCl (8.3 mM/min). The active fractions were collected (DEAE-Toyopearl). Aliquots of 2 M NaCl and 1 M potassium phosphate, pH 6.5 were added to the sample to final concentrations of 0.2 M NaCl and 50 mM potassium phosphate, pH 6.5, respectively, and then the sample was applied to a Con A-Sepharose column (Pharmacia Biotech; 5×5 mm) equilibrated with 50 mM potassium phosphate, pH 6.5, containing 0.2 M NaCl. After washing with 10 ml of the same buffer, the gel (about 100 μl bed volume) was transferred to two polypropylene micro test tubes (1.5 ml). To each tube, an aliquot (0.8 ml) of 50 mM potassium phosphate, pH 6.5, containing 0.2 M NaCl and 0.5 M α-methyl-D-mannoside was added, and the tubes were gently rotated at room temperature for 20 min. After centrifugation at 1300×*g* for 1 min, the supernatant was collected. The extraction was carried out three times. The combined extracts were concentrated to a final volume of 150–200 μl by using a Centriflo CF25 membrane concentrator (Amicon). The concentrate (Con A-Sepharose) was further purified by gel filtration on a TSK-GEL G3000SW column (Tosoh, Tokyo; 7.5×600 mm) equilibrated with 25 mM MES, pH 6.0, containing 0.2 M NaCl. The flow rate was 0.8 ml/min. The active fractions were collected, and concentrated to a final volume of 100–150 μl by using microconcentrators (Microcon 30, Amicon). The purified enzyme was stored at −80°C until use.

The protein concentrations of samples during the purification were determined with bicinchoninic acid protein assay reagent (Pierce). The concentrations of the purified enzyme were determined spectrophotometrically using $E^{1\%}_{280}$=12.4 [24]. UV-VIS spectra were obtained with a Shimadzu UV-2200 spectrophotometer equipped with a Shimadzu ultra micro cell holder, and we used 0.1 ml of samples for spectroscopic measurements.

2.3. Assay of dopamine β-monooxygenase activity

DβM activity was assayed at 25°C as the oxygen consumption rate using a Yellow Springs model 53 polarographic oxygen electrode equipped with a Shimadzu C-R4A integrator to amplify the electrode signals, or as the octopamine formation rate using the spectroscopic method described by Wallace et al. [5] and modified by Ljones et al. [23]. In both assays, the standard assay mixtures (2.7 ml for oxygen assay and 0.25 ml for octopamine assay) contained 10 mM tyramine, 10 mM ascorbate, 0.2 mM fumarate [15], 2 μM $CuSO_4$, 0.1 mg/ml catalase, and 50 mM MES buffer (pH 5.1 and ionic strength I=0.15). When the enzyme activities of crude extracts were assayed, *N*-ethylmaleimide was included in the standard assay mixtures (final concentration of 30 mM). To obtain kinetic parameters, tyramine concentration was varied from 0.1 to 10 mM.

2.4. Polyacrylamide gel electrophoresis

SDS-PAGE was performed on a 7.5% gel according to the method of Laemmli [25]. Samples were incubated with 2% SDS and 5% 2-mercaptoethanol in 62.5 mM Tris-HCl, pH 6.8, at 70–80°C for 3 min, and then subjected to SDS-PAGE. Proteins were stained with Coomassie brilliant blue R-250.

2.5. Molecular weight determination

The molecular weights of purified DβM were determined at 25°C by low-angle laser light scattering measurement combined with gel chromatography [26]. A TSK-GEL G4000SW$_{XL}$ column (7.8×300 mm), a UV-8011 uv detector, an LS-8000 low-angle laser light scattering photometer, all from Tosoh, and a Shimadzu RID-6A differential refractometer were used. The detectors were connected in tandem in this order. The buffer was 50 mM MES, pH 5.75, I=0.15, and the flow rate was 0.5 ml/min. The molecular mass standards used were albumin monomer and dimer (bovine serum, M_r=66 300 and 132 000, respectively), catechol 2,3-dioxygenase (*Pseudomonas putida*, M_r=140 000), and glutamate dehydrogenase (yeast, M_r=297 000); the former two proteins were purified [27,28], the third one was from Oriental Yeast, Tokyo.

2.6. Differential peptide mapping

Enzyme preparations (10 μg each) were lyophilized and then reduced at 37°C with 5 mM DTT in 7 M guanidine-HCl, 0.2 M Tris-HCl, pH 8.7, and 5 mM EDTA. After 20 min, an equal volume of 20 mM I-AEDANS was added to label free thiols. After 4 h of incubation at 37°C under nitrogen atmosphere, 2-mercaptoethanol (2 μl) was added to the mixture, and it was dialyzed against 1 liter of 50 mM sodium phosphate buffer, pH 7.5, at 4°C overnight. After dialysis, an equal volume of 8 M urea was added, and then each sample was digested with 1 μg of TPCK-trypsin at 25°C for 48 h. Peptide fragments were separated by reverse-phase HPLC on a Cosmosil 5C18 column (4.6×250 mm, Nacalai Tesque) at 25°C. Elution was carried out with a linear gradient of acetonitrile (0.575% per min and an initial concentration of 14%) containing 0.1% trifluoroacetic acid at a flow rate of 1 ml/min. Fluorescence at 480 nm was monitored with excitation at 340 nm by means of a Shimadzu RF-535 fluorescence detector.

To assign cysteine-containing peptide fragments, enzyme samples (70–100 μg) were labeled with I-AEDANS and digested by the same method. The main peptide peaks were collected, and the NH_2-terminal amino acid sequence of each peptide fragment was determined using a protein sequencer, Applied Biosystems Model 470A, with an on-line PTH amino acid analyzer, Model 120A.

2.7. Amino acid analysis

Amino acid analyses were carried out with a Hitachi L-8500 amino acid analyzer. The enzyme samples (about 10 μg) were hydrolyzed in the vapor phase of 6 M HCl containing 0.1% phenol at 110°C for 24 h in evacuated reaction vials from Waters Pico Tag work station. To determine the protein concentration of the samples used for absorption spectroscopy by amino acid analysis, γ-amino-*n*-butyric acid was used as an internal standard. We calculated the enzyme concentration using the obtained amino acid contents of Asp, Glu, Gly, Ala, Val, Ile, Leu, Phe, Lys, His, Arg, and Pro, and the respective amino acid contents per subunit of DβM deduced from cDNA, 42, 68, 41, 41, 39, 24, 59, 28, 16, 21, 34, and 43.

2.8. Circular dichroism spectroscopy

CD spectra were obtained using a Jasco spectropolarimeter, Model J-600, at 25°C in 5 mM MES, pH 6.5, containing 0.15 M NaCl. A quartz cell with light-path length of 0.1 cm was used and the protein concentrations were 0.1–0.4 mg/ml. The mean residue ellipticity, θ, was calculated using the average residue molecular weight of 112.3 [11].

3. Results

3.1. Purification of dopamine β-monooxygenase from a single adrenal gland

An example of purification of the enzyme from a single adrenal gland is given in Table 1. On DEAE-Toyopearl chromatography, the enzyme activity was eluted as a single peak

Table 1
Purification of dopamine β-monooxygenase from a single bovine adrenal gland

Purification step	Total volume (ml)	Total activity (units)	Total protein (mg)	Specific activity (units/mg)	Yield (%)
Crude extract	9.50	14.3	450	0.03	100
Polyethylene glycol	1.72	10.3	49.2	0.21	72
DEAE-Toyopearl	8.95	6.4	3.2	2.0	45
Con A-Sepharose	0.39	3.4	0.20	17.0	24
Gel filtration	0.22	2.2	0.13	17.0	15

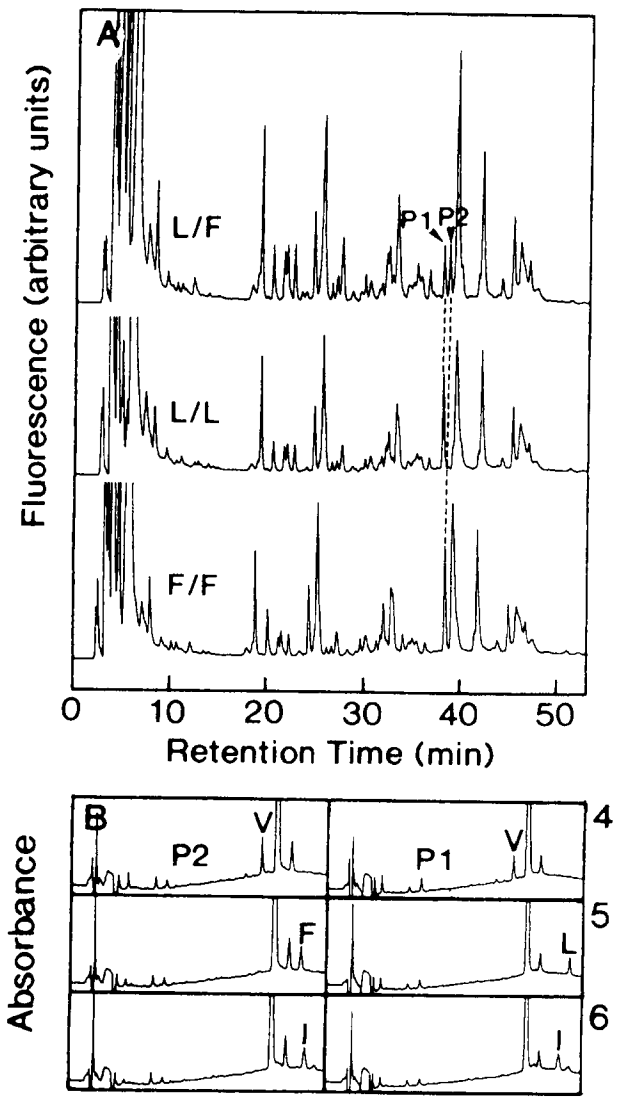

Fig. 1. Identification of a natural variant of dopamine β-monooxygenase in the enzyme preparations purified from individual adrenal glands. A: Reverse-phase HPLC analyses of tryptic fragments containing AEDANS-labeled cysteine(s) from three different preparations on a column of Cosmosil 5C18. B: Steps 4, 5, and 6 of the N-terminal sequencing for the peptides P1 and P2. The N-terminal 17 residues were unambiguously determined for both peptides.

with about 0.25 M NaCl (data not shown). Gel filtration on a TSK-GEL G3000SW removed trace amounts of contaminated proteins with smaller Stokes radii from the preparation obtained by Con A-Sepharose chromatography. The overall recovery of the enzyme activity was about 15%, and a sufficient amount of DβM (100–300 μg) for characterization was obtained.

We purified DβM from each of 18 individual adrenal glands. The specific activities of the purified enzyme were in the range of 15–40 μmol/min/mg, comparable to that of the enzyme purified in bulk (about 20 μmol/min/mg). It took maximally 8 h to obtain pure DβM from a single adrenal gland by the present procedure.

3.2. *Identification of a novel variant*

To examine mutations in the primary structure of each preparation purified separately from 18 single adrenal glands, all disulfides of each preparation were reduced with DTT, and then the resulting free thiols were labeled with I-AEDANS. After TPCK-trypsin digestion, cysteine-containing peptides from each enzyme sample were separated by reverse-phase HPLC (Fig. 1A). Comparison of the chromatograms obtained for the 18 samples revealed that the two peptides (P1 and P2 in Fig. 1) showed variations in amount among the DβM preparations: seven samples contained an equal amount of the two peptides (the upper chromatogram in Fig. 1A); four preparations contained only P1 (the middle chromatogram), while the remaining seven samples showed only P2 (the lower chromatogram).

The N-terminal sequence of P1 was determined to be APDVLIPGQQTTYWC*YV, where C* is an AEDANS-labeled cysteine residue and it could not be detected as a peak upon usual PTH-amino acid analysis by a protein sequencer. This sequence was identical to sequence 204–220 deduced from cDNAs. As shown in Fig. 1B, the fifth residue of the N-terminal sequence of P2 was determined to be F, different from the L of P1, and all other residues were identical to those of P1.

These results indicate the presence of a novel variant of DβM with F as residue 208, and that there are three phenotypes of DβM in Japanese black cattle, viz. L208 homoenzyme (L/L), F208 homoenzyme (F/F), and L208/F208 heteroenzyme (L/F).

3.3. *Characterization of the variant dopamine β-monooxygenase*

DβM preparations purified in bulk exhibit three protein bands on SDS-PAGE under reducing conditions [9]. As shown in Fig. 2A, all of the wild-type, variant and heteroenzyme (L/L, F/F, and L/F) were separated into one major band of 76 kDa and two additional bands of 79 and 73 kDa. The result shows that the heterogeneity of DβM is due to post-transcriptional modifications.

The molecular weights of L208 homoenzyme, F208 homoenzyme, and L208/F208 heteroenzyme were determined to be 2.3×10^5, 2.2×10^5, and 2.5×10^5, respectively, by low-angle laser light scattering photometry coupled with high-performance gel chromatography (Fig. 2B). Since the subunit molecular weight estimated from cDNA is 6.5×10^4, the result shows that all three types of DβM are a tetramer.

The conformations of the three types of DβM were investigated by CD spectroscopy (Fig. 2C). The observed CD spectra were similar to each other: they showed a deep trough at 214 nm and a shallow trough at 242 nm. No significant difference was found among the absorption spectra of the three phenotypes (Fig. 2D). They showed a peak at 278 nm with the molar absorption coefficient of about 8.65×10^4 $M^{-1}\cdot cm^{-1}$. The value agreed well with that commonly used for bovine DβM (8.99×10^4 $M^{-1}\cdot cm^{-1}$) [24], and significantly larger than that calculated on the basis of the bovine cDNA derived sequences (6.49×10^4 $M^{-1}\cdot cm^{-1}$) [29].

No significant difference was found among the amino acid compositions of the wild-type, variant, and heteroenzyme. The compositions agreed with those predicted from the nucleotide sequences [10–12] within experimental errors (data not shown).

3.4. *Kinetic parameters*

To compare kinetic properties of the three types of DβM,

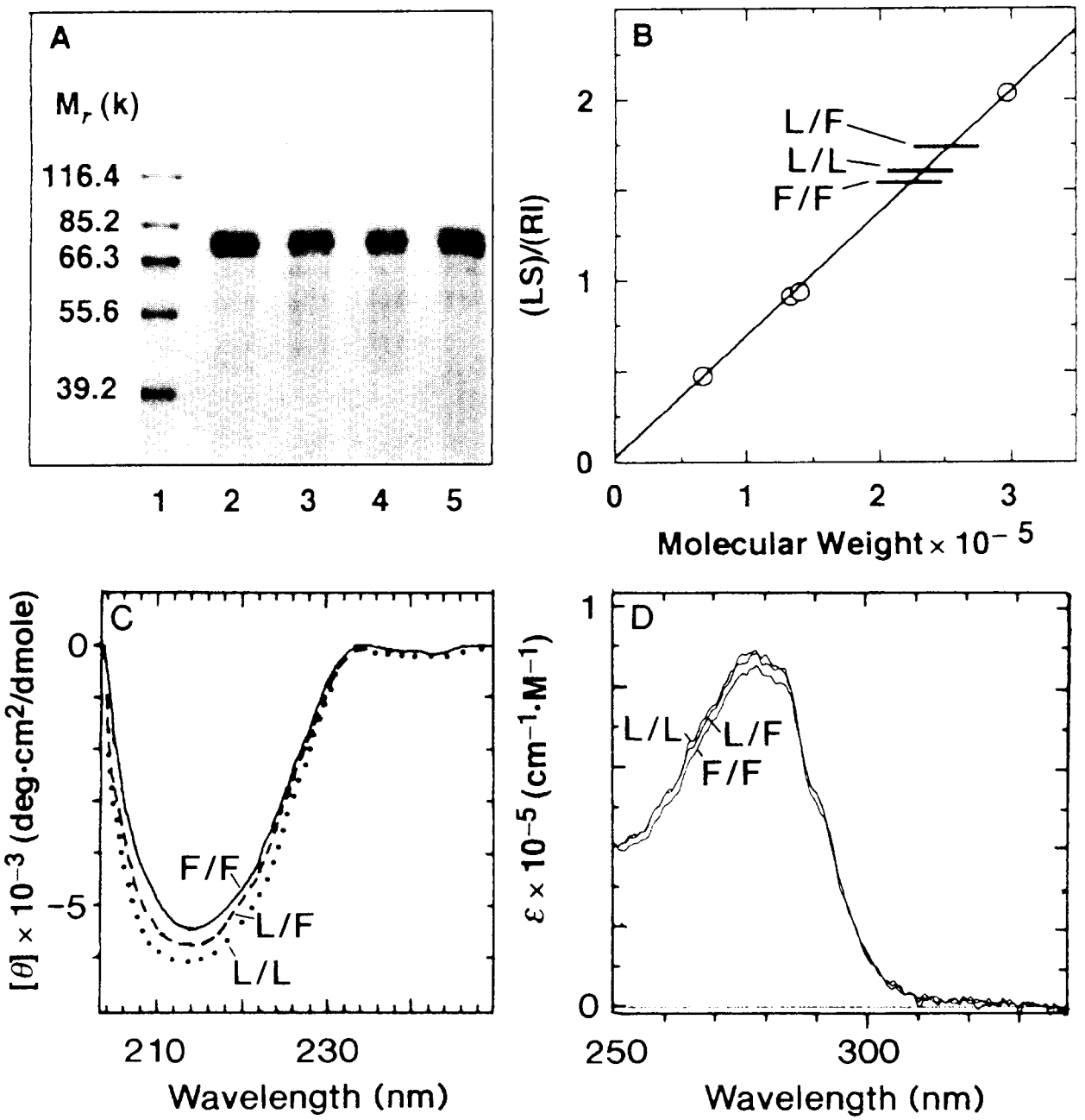

Fig. 2. Structural and spectral characterization of the wild-type (L/L), variant (F/F), and heteroenzymes (L/F) of dopamine β-monooxygenase. A: SDS-PAGE of the marker proteins (lane 1), L/L enzyme (1.0 μg, lane 2), F/F enzyme (1.0 μg, lane 3), L/F enzyme (1.0 μg, lane 4), and mixture of L/L and F/F (both 0.5 μg, lane 5). B: Molecular weight determination. The ratio of the output of the light scattering photometer (LS) to that of the differential refractometer (RI) was plotted against molecular weight. The enzyme concentrations at the elution peak were 4.5 μg/ml for L/L, 5.8 μg/ml for F/F, and 6.5 μg/ml for L/F. C: Far-UV circular dichroism spectra. The protein concentrations were 0.4 mg/ml for the two homoenzymes and 0.1 mg/ml for the heteroenzyme. D: Absorption spectra. Spectra were obtained in 10 mM MES, pH 6.5, containing 0.2 M NaCl.

we performed kinetics for each of the 18 DβM preparations (Fig. 3). All of the preparations showed a hyperbolic dependence of the initial velocity on tyramine concentration. We could not found any significant difference in kinetic properties among the three phenotypes of DβM.

4. Discussion

In the present study we have isolated a novel variant of bovine dopamine β-monooxygenase arising from a $Leu^{208} \rightarrow Phe$ substitution. This is probably due to a point mutation, CTC→TTC, at position 634 in the DβM gene, although a mutation of CTC→TTT is also possible. The variant subunit was found in 14 out of 18 adrenal glands examined, suggesting an allelic polymorphism of the DβM gene in Japanese black cattle. Seven adrenal glands were heterozygous for the variant, and all of them contained an equal amount of the variant (F208) and the wild-type (L208) subunit.

We searched five DβM preparations purified in bulk for the variant. Various amounts of the variant (10–50%) were found.

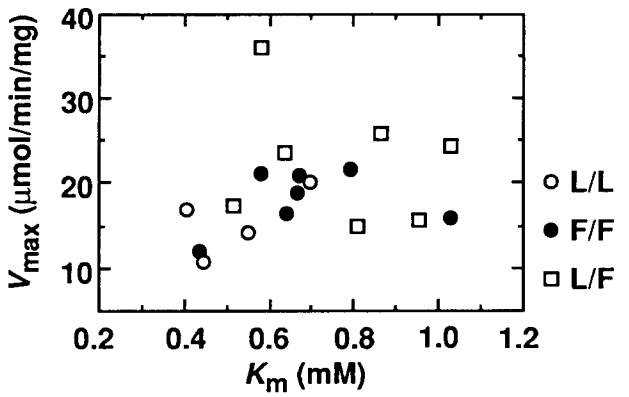

Fig. 3. Kinetic parameters for dopamine β-monooxygenase preparations individually purified from a single adrenal gland. The activities were assayed at 25°C in 50 mM MES, pH 5.1, $I=0.15$. The reactions were initiated by adding 0.2 μg of the enzyme into 0.25 ml of reaction mixtures. The values of kinetic parameters were obtained from [S]/v versus [S] plots, where [S] is the tyramine concentration and v the initial velocity.

We purified the enzyme from a commercially available DβM preparation (Sigma, Lot 95H7100), and examined the variant content. This sample contained only the wild-type protein (L208). These results imply that chromatographic methods used for DβM purification cannot separate the three phenotypes of L/L, L/F, and F/F.

Since the residue Y216 is essential for the activity [17] and Phe is a bulky aromatic residue, the substitution from aliphatic Leu to Phe at position 208 near Y216 may be expected to affect the active site environment of DβM. However, the variant exhibited essentially identical kinetic properties compared to those of the wild-type enzyme. The result suggests that residue 208 does not participate in the active site structure, or that the structure around the active site is flexible to accommodate the perturbation induced by the substitution.

Two allelic forms of human dopamine β-monooxygenase with either alanine or serine at position 304 were cloned from a cDNA library [30], and it was reported that 304S DβM was one-tenth less active than 304A DβM [31]. However, the recent report shows that the recombinant 304S is not significantly different from the recombinant 304A in kinetic and other enzyme properties [32]. Since human plasma contains about 10 μg/ml DβM [33], it is possible to screen genetic variants of DβM not only by genetic technology but also by protein chemical methods used in the present study. A rapid purification of DβM from human plasma (1–10 ml) with high yields is now under development in our laboratory.

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References

[1] Winkler, H., Apps, D.K. and Fischer-Colbrie, R. (1986) Neuroscience 18, 261–290.
[2] Stewart, L.C. and Klinman, J.P. (1988) Annu. Rev. Biochem. 57, 551–592.
[3] Zhou, Q.-Y., Quaife, C.J. and Palmiter, R.D. (1995) Nature 374, 640–643.
[4] Thomas, S.A., Matsumoto, A.M. and Palmiter, R.D. (1995) Nature 374, 643–646.
[5] Wallace, E.F., Krantz, M.J. and Lovenberg, W. (1973) Proc. Natl. Acad. Sci. USA 70, 2253–2255.
[6] Craine, J.E., Daniels, G.H. and Kaufman, S. (1973) J. Biol. Chem. 248, 7838–7844.
[7] Lewis, E.J. and Asnani, L.P. (1992) J. Biol. Chem. 267, 494–500.
[8] Robertson, J.G., Adams, G.W., Medzihradszky, K.F., Burlingame, A.L. and Villafranca, J.J. (1994) Biochemistry 33, 11563–11575.
[9] Joh, T.H. and Hwang, O. (1987) Ann. NY Acad. Sci. 493, 342–350.
[10] Taljanidisz, J., Stewart, L., Smith, A.J. and Klinman, J.P. (1989) Biochemistry 28, 10054–10061.
[11] Lewis, E.J., Allison, S., Fader, D., Claflin, V. and Baizer, L. (1990) J. Biol. Chem. 265, 1021–1028.
[12] Wang, N., Southan, C., DeWolf, W.E., Jr., Wells, T.N.C., Kruse, L.I. and Leatherbarrow, R.J. (1990) Biochemistry 29, 6466–6474.
[13] Saxena, A., Hensley, P., Osborne, J.C., Jr. and Fleming, P.J. (1985) J. Biol. Chem. 260, 3386–3392.
[14] Dhawan, S., Hensley, P., Osborne, J.C., Jr. and Fleming, P.J. (1985) J. Biol. Chem. 261, 7680–7684.
[15] Stewart, L.C. and Klinman, J.P. (1991) J. Biol. Chem. 266, 11537–11543.
[16] Ishida, T., Narita, M., Nozaki, M. and Horiike, K. (1996) J. Biochem. 120, 346–352.
[17] DeWolf, W.E., Jr., Carr, S.A., Varrichio, A., Goodhart, P.J., Mentzer, M.A., Roberts, G.D., Southan, C., Dolle, R.E. and Kruse, L.I. (1988) Biochemistry 27, 9093–9101.
[18] DeWolf, W.E., Jr., Chambers, P.A., Southan, C., Saunders, D. and Kruse, L.I. (1989) Biochemistry 28, 3833–3842.
[19] Farrington, G.K., Kumar, A. and Villafranca, J.J. (1990) J. Biol. Chem. 265, 1036–1040.
[20] Reedy, B.J. and Blackburn, N.J. (1994) J. Am. Chem. Soc. 116, 1924–1931.
[21] Eipper, B A., Quon, A.S.W., Mains, R.E., Boswell, J.S. and Blackburn, N.J. (1995) Biochemistry 34, 2857–2865.
[22] Colombo, G., Papadopoulos, N.J., Ash, D.E. and Villafranca, J.J. (1987) Arch. Biochem. Biophys. 252, 71–80.
[23] Ljones, T., Skotland, T. and Flatmark, T. (1976) Eur. J. Biochem. 61, 525–533.
[24] Skotland, T. and Ljones, T. (1977) Int. J. Pept. Protein Res. 10, 311–314.
[25] Laemmli, U.K. (1970) Nature 227, 680–685.
[26] Hayashi, Y., Matsui, H. and Takagi, T. (1989) in: Methods in Enzymology (Fleischer, S. and Fleischer, B., Eds.), Vol. 172, pp. 514–528, Academic Press, San Diego, CA.
[27] Janatova, J., Fuller, J.K. and Hunter, M.J. (1968) J. Biol. Chem. 243, 3612–3622.
[28] Kobayashi, T., Ishida, T., Horiike, K., Takahara, Y., Numao, N., Nakazawa, A., Nakazawa, T. and Nozaki, M. (1995) J. Biochem. 117, 614–622.
[29] Robertson, J.G., Desai, P.R., Kumar, A., Farrington, G.K., Fitzpatrick, P.F. and Villafranca, J.J. (1990) J. Biol. Chem. 265, 1029–1035.
[30] Kobayashi, K., Kurosawa, Y., Fujita, K. and Nagatsu, T. (1989) Nucleic Acids Res. 17, 1089–1102.
[31] Ishii, A., Kobayashi, K., Kiuchi, K. and Nagatsu, T. (1991) Neurosci. Lett. 125, 25–28.
[32] Li, B., Tsing, S., Kosaka, A.H., Nguyen, B., Osen, E.G., Bach, C., Chan, H. and Barnett, J. (1996) Biochem. J. 313, 57–64.
[33] Frigon, R.P. and Stone, R.A. (1978) J. Biol. Chem. 253, 6780–6786.