

Characterization of a novel human catechol-*O*-methyltransferase mutant with triplet point mutations

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Abstract. Human catechol-*O*-methyltransferase (COMT, EC 2.1.1.6) catalyzes the transfer of the methyl group to a variety of endogenous and exogenous catechol substrates using *S*-adenosyl-*L*-methionine as the methyl donor. This enzymatic *O*-methylation plays an important role in the inactivation of biologically-active and toxic catechols. A number of studies in recent years have sought to characterize the polymorphism of human COMT, and also to determine the catalytic activity of polymorphic enzymes. We report here the identification of a new haplotype of the human *COMT* gene with triplet point mutations, which encodes the D51G/S60F/K162R mutant of the soluble COMT and the D101G/S110F/K212R mutant of the membrane-bound COMT. Kinetic analysis showed that these new COMT variants had essentially the same kinetic characteristics and catalytic activity as the wild-type COMTs for the *O*-methylation of 2-hydroxyestradiol and 4-hydroxyestradiol *in vitro*, but they have a significantly reduced thermostability at 37°C. In addition, the mutant enzymes have different binding affinities for *S*-adenosyl-*L*-methionine compared with the wild-type COMTs. In agreement with our biochemical observations, molecular modeling studies also showed that the variant human COMT proteins shared nearly the same overall structures as the wild-type proteins. The binding energy values of the mutant COMTs in complex with catechol estrogen substrates were similar to those of the wild-type COMTs bound with the same substrates.

Introduction

Human catechol-*O*-methyltransferase (COMT; EC 2.1.1.6) catalyzes the transfer of the methyl group to a wide variety of endogenous and exogenous catechol substrates by using *S*-adenosyl-*L*-methionine (AdoMet) as the methyl donor (1-8). In the central nervous system, COMT metabolically deactivates catecholamine neurotransmitters (dopamine and norepinephrine) through *O*-methylation. The human COMT exists in two different forms, the soluble form (S-COMT) and the membrane-bound form (MB-COMT), which are encoded by a single gene (8,9). A decrease in the methylation of catecholamine neurotransmitters due to the presence of a thermolabile S-COMT variant (V108M) has been associated with an increased risk for Parkinson's disease (3,9), the development of a chronic pain condition (10-12), and also a number of mental disorders (12-21). In addition to catecholamine neurotransmitters, the endogenous catechol estrogens, such as 2- and 4-hydroxyestradiol (2-OH-E₂ and 4-OH-E₂), which are major oxidative metabolites of 17β-estradiol (E₂) formed by cytochrome P450 isoforms in humans (22,23), are also rapidly *O*-methylated by COMT, in a manner analogous to the *O*-methylation of catecholamines (21). A number of studies have demonstrated that metabolic *O*-methylation provides effective inactivation/detoxification of the procarcinogenic catechol estrogen intermediates. The major *O*-methylation product of 2-OH-E₂ (2-methoxyestradiol) has strong apoptotic, antiangiogenic, and anticancer actions [reviewed in refs. (3,24)]. Hence, metabolic *O*-methylation of catechol estrogens may not only inactivate the catechol estrogen intermediates, but may also simultaneously produce 2-methoxyestradiol that has significant anticancer activity (24). These two concurrent processes are thought to be beneficial for reducing the risk of estrogen-induced cancers (24). In line with this suggestion, many epidemiological studies have shown that women, homozygous with a thermolabile variant (V108M) of S-COMT, have an increased risk of estrogen-associated cancers (25-32), thus providing support for this interesting hypothesis.

Given the potentially important biological consequences associated with a reduced COMT catalytic activity *in vivo*, a number of studies in recent years have sought to investigate the polymorphism of the human *COMT* gene, as well as its effect on the catalytic activity of the enzyme (33-37). At present, a total of 14 polymorphisms (summarized in Table I)

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Abbreviations: COMT, catechol-*O*-methyltransferase; E₂, 17β-estradiol; 2-OH-E₂ and 4-OH-E₂, 2- and 4-hydroxyestradiol, respectively; AdoMet, *S*-adenosyl-*L*-methionine; AdoHcy, *S*-adenosyl-*L*-homocysteine

Key words: catechol-*O*-methyltransferase, catechols

Table I. The COMT polymorphisms listed in the NCBI SNP database (<http://www.ncbi.nlm.nih.gov/SNP>).

Amino acid positions (S/MB)	Amino acid residue		Effect on the catalytic activity of the enzyme <i>per se</i>
	Wild-type	Mutant	
-/9	L	F	Unknown (unpublished)
-/34	C	S	Unknown (unpublished)
12/62	H	H ^a	No change (32)
22/72	A	S	Lower catalytic activity and reduced thermostability (33)
23/73	Q	Q ^a	No change
42/92	V	M	Unknown (unpublished)
52/102	A	T	Reduced thermostability (33)
62/112	L	L ^a	No change
84/134	A	A ^a	No change
86/136	L	L ^a	No change (32)
96/146	A	V	Unknown (unpublished)
108/158	V	M	Reduced thermostability (17, 29-32)
149/199	P	L	Unknown (unpublished)
153/203	L	L ^a	No change

^aThese nucleic acid mutations will not alter the amino acids that are encoded by the altered codons.

are listed in the NCBI SNP database for the coding regions (exons) of the human *COMT* gene, and each of them are single point mutations. Among them, only three of the *COMT* mutants have been studied for their enzymatic activity and functional differences with the wild-type *COMT*. The V108M mutant of human S-*COMT* was found to retain nearly the same catalytic ability for the *O*-methylation of catechol estrogen substrates as the wild-type *COMT*, but it had a reduced thermostability (32-36). In comparison, the A22S mutant of the human S-*COMT* had a lower catalytic activity and also reduced thermostability (37).

In the present study, we identified a new haplotype of the human *COMT* gene. In addition, we have selectively expressed the wild-type and mutant human S- and MB-*COMT*s in *Escherichia coli* (*E. coli*) for comparing their biochemical characteristics for the *O*-methylation of endogenous catechol estrogens. Using S-*COMT* as an example, we additionally conducted computational molecular modeling studies to compare the structural and catalytic differences between the wild-type and variant enzymes.

Materials and methods

Chemicals. 2-OH-E₂, 4-OH-E₂, isopropylthio-β-*D*-galactoside (IPTG), 1,4-dithiothreitol (DTT) and phenylmethylsulfonyl fluoride (PMSF), *S*-adenosyl-*L*-methionine (AdoMet) were purchased from Sigma-Aldrich (St. Louis, MO). (Methyl-³H) AdoMet (specific activity, 11.2-13.5 Ci/mmol) was obtained from Perkin Elmer (Waltham, MA). All solvents used in this study were of HPLC grade or higher and were obtained from Fisher Scientific Co. (Springfield, NJ).

Cloning of human S-*COMT* and MB-*COMT* cDNAs. The human liver cDNA library, obtained from Stratagene (La Jolla, CA), was generated from pooled normal liver tissues

from two females, and was used as a template for cloning the human *S-COMT* and *MB-COMT* cDNAs. For PCR, the 5' complementary forward primers (5'-CAA CAT ATG CCG GAG GCC CCG-3' and 5'-GCA TAT GCC GGA GGC CCC GCC TC-3') were used for S-*COMT* and MB-*COMT*, respectively, along with a common 3' reverse primer (5'-CAG GAT CCT CAG GGC CCT GCT-3'). These primers were specifically designed to append the sequence that contains a 5' *NdeI* restriction site and a 3' *BamHI* restriction site (Fig. 1, lower panel). The resulting 655-bp fragment for *S-COMT* and the 815-bp fragment for *MB-COMT* amplified by PCR were eluted using a gel extract kit (Qiagen, Valencia, CA), and they were then ligated to the pGEM T vector (Promega, Madison, WI) using T4 ligase (Invitrogen, Carlsbad, CA). The products of the ligation reactions were transformed into the chemically competent *E. coli* TOP-10F' cells (Invitrogen, Carlsbad, CA), and the transformed bacteria were then selected with ampicillin (50 μg/ml) on LB agar plates. The plasmids were purified using a Miniprep purification kit (Qiagen, Valencia, CA). The entire *S-COMT* and *MB-COMT* cDNA sequences were determined for verification. Note that three different types of DNA polymerase, i.e., ExTaqDNA polymerase (Takara, Madison, WI), PfuTurbo DNA polymerase (Stratagene, La Jolla, CA), and HotStarTaq DNA polymerase (Qiagen, Valencia, CA), all of which have high fidelity and proof-reading capability, were used for the PCR for cloning the human *S-COMT* and *MB-COMT* cDNAs. The resulting cDNA products from three different DNA polymerases were sequenced and the sequences matched each other. In addition, the sequences of the overlapping regions of the *S-COMT* and *MB-COMT* cDNAs also matched each other.

The plasmids were then restriction-digested with *NdeI* and *BamHI* and ligated into *NdeI* and *BamHI*-digested pET12a vector (Novagen, Madison, WI). The recombinant

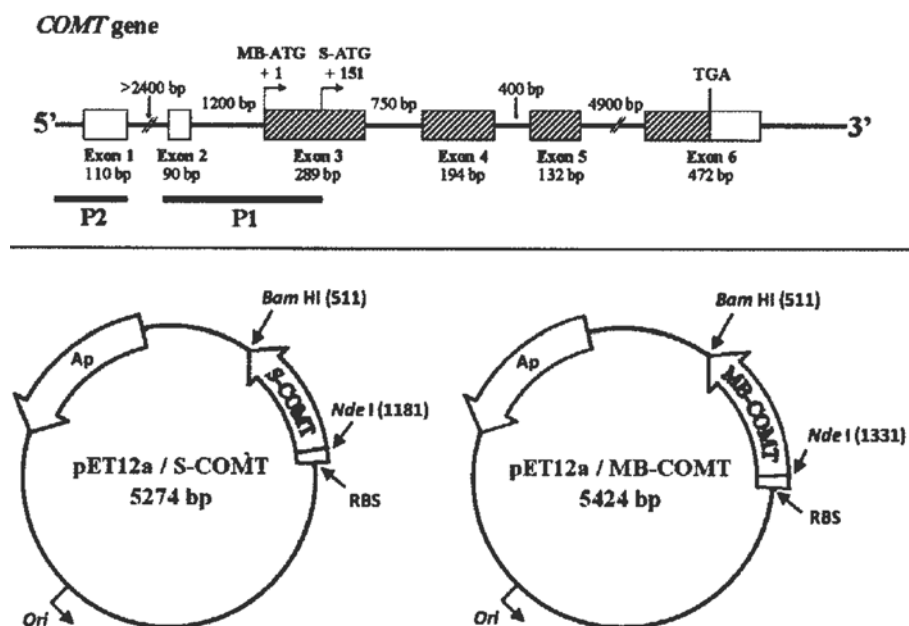


Figure 1. Upper panel: Structure of the human COMT gene. The boxes represent exons and the thin lines between the boxes represent introns. The hatched boxes indicate protein-coding regions. The size of each exon and intron is as indicated. The positions of the initiation codons for the transcription of S-COMT and MB-COMT mRNAs are indicated as S-ATG and MB-ATG. The two known promoters, P1 and P2, are shown by black bars. Note that the P1 promoter for transcription of S-COMT overlaps with the initiation codon and part of the coding sequence for MB-COMT. Lower panel: Construction of the pET12a/S-COMT and pET12a/MB-COMT expression vectors based on the vector pET12a. The S-COMT or MB-COMT cDNA was cloned into the *Nde*I and *Bam*HI sites of pET12a to form the pET12a/S-COMT or pET12a/MB-COMT expression vectors. Each of the expression vectors was under the control of the T7 promoter and lacO-operator. Expression was induced by the addition of 0.5 mM isopropylthio- β -D-galactoside.

DNAs were introduced into chemically competent *E. coli* BL21 (DE3) (Novagen, Madison, WI) according to the procedures recommended by the manufacturer, and the transformed cells were selected with ampicillin (50 μ g/ml) on LB agar plates.

In vitro site-directed mutagenesis. The cDNAs of the cloned mutant human S-COMT (D51G, S60F and K162R) and MB-COMT (D101G, S110F and K212R) were used as templates to generate the corresponding wild-type cDNAs. The mutations were corrected by using a PCR-based site-directed mutagenesis using the QuikChange multisite-directed mutagenesis kit (Stratagene, La Jolla, CA). The site-directed mutagenesis was carried out according to the procedures recommended by the manufacturer with the following primers: 5'-GGCAAGATCGTGGACGCCGTGATTC-3' for D51/101G, 5'-CACCAGCCCTCCGTGCTGCTGGAGC-3' for S60/110F, and 5'-CTGCTGCGGAAGGGGACAGTGTAC-3' for K162/212R. The sequences of reconstructed plasmid DNAs were confirmed by DNA sequencing.

Bacterial expression of recombinant human COMTs. For the expression of the recombinant human S- and MB-COMT proteins in *E. coli* BL21 (DE3, expressing T7 polymerase), positive clones were first cultured in the LB medium supplemented with ampicillin (50 μ g/ml) overnight at 37°C. The culture broth was then inoculated into 300 ml fresh LB medium supplemented with ampicillin and incubated at 37°C with vigorous shaking until the optical density reading of the bacterial culture mixture achieved \sim 0.6 (at $\lambda=600$ nm). The culture was then induced with isopropylthio- β -D-galactoside (at a final concentration of 0.5 mM) and cultured for another

3 h. The cells were collected by centrifugation and were then sonicated in ice-cold lysis buffer (50 mM Tris-HCl, pH 7.5 + 200 mM NaCl). After the addition of 5 mM 1,4-dithiothreitol and 1 mM phenylmethylsulfonyl fluoride to the crude homogenates, they were centrifuged at 10,000 \times g for 10 min at 4°C. The supernatants were then subjected to column purification or directly stored at -80°C.

Assay of the enzyme activity and thermostability of recombinant human COMTs in vitro. The catalytic activity of the wild-type and mutant human recombinant S- and MB-COMTs was determined at 37°C as described earlier (38). The reaction mixtures consisted of the recombinant COMT protein (at 16.2 μ g/ml for S-COMT or 17.1 μ g/ml for MB-COMT), 1.2 mM MgCl₂, 100 μ M AdoMet (containing 0.5 mCi [methyl-³H]AdoMet), 1 mM 1,4-dithiothreitol, and 2-OH-E₂ or 4-OH-E₂ as substrate (at 10 μ M or as indicated) in Tris-HCl buffer (50 mM, pH 7.4). The final volume of the reaction mixture was 300 μ l. The reaction was initiated by the addition of recombinant human COMT protein and carried out at 37°C for 15 min. To test the thermo-stability of the mutant and wild-type COMTs, the enzymes were first preincubated at 37°C for the indicated length of time immediately before testing their catalytic activity for the O-methylation of 2-OH-E₂ (at 10 μ M). The reaction was arrested by immediately placing the tubes on ice and followed by addition of 500 μ l ice-cold saline. The reaction mixtures were extracted with 5 ml ethyl acetate for the methylated catechol products. After centrifugation at 1000 g for 10 min, portions of the organic extracts were measured for radioactivity content with a liquid scintillation analyzer (Packard Tri-CARB 2900TR, Downers Grove, IL). The rate

of methylation of a substrate was expressed as 'nmol of methylated product formed/mg of human COMT protein/minute' (abbreviated as 'nmol/mg protein/min'). The kinetic parameters (K_M and V_{MAX} values) were calculated by using the curve regression method of the SigmaPlot program.

Construction of the homology models for human wild-type and mutant S-COMTs. Homology modeling was performed using the InsightII modeling program (Version 2005, Accelrys Inc., San Diego, CA) on a Dell Precision 690 workstation installed with Red Hat Enterprise Linux WS4.0 operating system (Red Hat Inc., Raleigh, NC). The energy minimization and molecular dynamics simulation were performed with Discovery Studio modeling program (Version 1.7, Accelrys Inc., San Diego, CA). The *CHARMM* force field was used for energy minimization and dynamics simulation.

The primary sequences of human S-COMT (GeneBank accession no. NM007310) and rat S-COMT (GeneBank accession no. BC081850) were obtained from the NCBI database. Sequence alignments were performed by using the Homology Modeling module of InsightII, which showed a $\geq 80\%$ sequence similarity. The homology model of the human S-COMT was constructed according to the rat S-COMT (PDB code: 1VID) by using the Modeler in the Homology Modeling module of InsightII. The substrate 3,5-dinitrocatechol, AdoMet, Mg^{2+} ion, and the crystallographic H_2O that coordinates with Mg^{2+} were included in the homology model. The simulation was carried out using the Standard Dynamics Cascade protocol in the Discovery Studio. For energy minimization, the steepest descent method was employed first to a 10 kcal/(mol \AA) root mean square gradient and followed by the Polak and Ribiere conjugate gradient method until the final convergence criterion achieved 0.01 kcal/(mol \AA) gradient. Then the whole system was heated from 50 to 300 K in 2 ps and equilibrated in 300 K for 100 ps. One hundred conformations were collected in 20-ps production phase at 300 K. The conformation with the lowest potential energy was further minimized and used for binding energy analysis. 3,5-Dinitrocatechol, Mg^{2+} , H_2O , AdoMet and key residues in the catalytic site (D141, K144, D169, N170 and E199) were constrained during the simulation process.

It should be noted that the structure of the D51G/S60F/K162R mutant human S-COMT was built with the Build Mutant function of the Homology Modeling module by using two different methods: one was based on the homology model of the wild-type human S-COMT that was built in the present study and the other was based on the crystallographic structure of the rat S-COMT (PDB code: 1VID). The same simulation process was carried out when we built the structures of the mutant or wild-type S-COMT.

Calculation of the binding energy values ($\Delta E_{binding}$) of S-COMTs with substrates. The structures of 2-OH- E_2 and 4-OH- E_2 were built with the Builder module of InsightII based on the X-ray structure of E_2 (PDB code: 1ERE) and minimized with *CHARMM* force field. The catechol ring of the substrates was superimposed onto the catechol ring of 3,5-dinitrocatechol. The simulation was carried out with the

Standard Dynamics Cascade protocol in Discovery Studio. The same simulation cascade was carried out as the cascade that was used for building the human COMT in complex with 3,5-dinitro-catechol as ligand. One hundred conformations were collected in the 20-ps production phase at 300 K. The conformation with the lowest potential energy was further minimized and used for the Binding Energy Calculation protocol. The backbone of the protein, key residues in the catalytic site (D141, K144, D169, N170 and E199), the catechol ring of the substrate, AdoMet, Mg^{2+} , and H_2O were constrained during the whole simulation process. $\Delta E_{binding}$ was calculated with the following equation: $\Delta E_{binding} = E_{complex} - (E_{COMT} + E_{substrate})$, where $E_{complex}$ is the potential energy for the complex of human COMT with its substrate, E_{COMT} is the potential energy of the enzyme itself and $E_{substrate}$ is the potential energy for the substrate itself.

Results

Cloning of the mutant human S- and MB-COMT cDNAs. In the present study, the human S- and MB-COMT cDNAs were selectively amplified with PCR using the human liver cDNA library as a template. After the recombinant plasmids containing the cDNA for human S-COMT or MB-COMT were enzymatically digested, gel electrophoresis of the digests revealed that the DNA fragment bands matched the expected sizes of 655 and 815 bp, respectively (data not shown). The full-length sequence analysis of the cloned cDNAs was performed to determine their sequences. By comparing with sequences of NM_000754 and NM_007310, which were used as standards for the wild-type human S-COMT and MB-COMT, respectively, we found that the sequences were changed at three places for S-COMT and MB-COMT cDNAs, and the changes were at the same positions for the two of them. As noted earlier, three different types of DNA polymerase, each with high fidelity and proof-reading capability, were used in this study for the PCR for cloning the human S-COMT and MB-COMT cDNAs. The resulting cDNA products from three different DNA polymerases were sequenced and the same sequence was obtained. Notably, since it is known that a single gene [localized to chromosome 22, band q11.2 (8,9)] encodes human S- and MB-COMT proteins by using two separate promoters (6), we believe that the triplet point mutations of the human S- and MB-COMT cDNAs were not artificial errors produced during the cloning procedures, as the exact same triplet point mutations were seen in the cDNAs of S-COMT (D51G/S60F/K162R) and MB-COMT (D101G/S110F/K212R). The likelihood of their occurrence as a result of experimental errors was very low.

To obtain the wild-type human S- and MB-COMT cDNAs, we carried out PCR-based site-directed mutagenesis based on the mutant human S- and MB-COMT cDNAs. The full-length DNA sequences were determined twice and compared with the known wild-type human *COMT* gene. The DNA sequencing results confirmed their right sequences.

Expression of the mutant and wild-type COMT proteins in E. coli. The *E. coli* BL21 (DE3) cells were transformed with the recombinant expression vector pET12a containing the gene

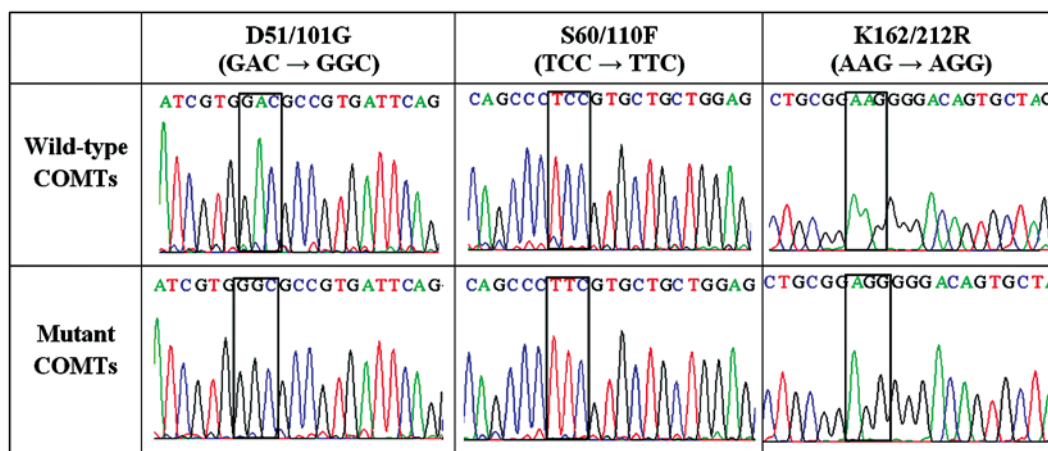


Figure 2. Sequencing results of the wild-type and haplotype human S- and MB-COMTs.

Table II. A comparison of the kinetic parameters of the mutant forms of the human S- and MB-COMTs with those of wild-type human S- and MB-COMTs.

Genotype	Substrate (μM)	AdoMet (μM)	K_M (μM)	V_{MAX} (nmol/mg/min)	V_{MAX}/K_M
Wild-type COMT					
Enzyme:					
S-COMT	2-OH-E ₂ (0–80)	100	3.6	9.7	2.7
	4-OH-E ₂ (0–80)	100	4.5	3.5	0.8
MB-COMT	2-OH-E ₂ (0–80)	100	3.2	16.3	5.1
	4-OH-E ₂ (0–80)	100	6.4	14.3	2.2
Mutant COMT (D51G/S60F/K162R)					
Enzyme:					
S-COMT	2-OH-E ₂ (0–80)	100	3.3	8.7	2.6
	4-OH-E ₂ (0–80)	100	4.5	3.5	0.8
MB-COMT	2-OH-E ₂ (0–80)	100	2.6	16.1	6.2
	4-OH-E ₂ (0–80)	100	5.0	14.1	2.8
Wild-type COMT					
Enzyme:					
S-COMT	2-OH-E ₂ (10)	0–400	113.6 ^a	18.3 ^a	-
	4-OH-E ₂ (10)	0–400	145.3 ^a	17.1 ^a	-
Mutant COMT (D51G/S60F/K162R)					
Enzyme:					
S-COMT	2-OH-E ₂ (10)	0–400	79.5 ^a	18.1 ^a	-
	4-OH-E ₂ (10)	0–400	146.7 ^a	9.6 ^a	-

^aTo determine the apparent K_M and V_{MAX} values of AdoMet for the *O*-methylation catalyzed by S-COMT and MB-COMT, a fixed concentration (at 10 μM) of 2-OH-E₂ or 4-OH-E₂ was used as a substrate, and different concentrations of AdoMet (at 0, 12.5, 25, 50, 100, 200, and 400 μM) were used as the methyl donor.

encoding either the mutant or wild-type human S-COMT or MB-COMT. Following induction with 0.5 mM isopropylthio- β -*D*-galactoside, the bacteria that abundantly expressed the desired protein were harvested by centrifugation. The resulting cell pellets were lysed and analyzed using 12% SDS-PAGE, followed by Western blot analysis using polyclonal rabbit antibodies against the human wild-type COMT and donkey anti-rabbit IgG antiserum (conjugated to horseradish peroxidase).

A band with a molecular mass of ~24 kD was detected for mutant and wild-type S-COMT proteins, and a band of ~30 kD was detected for the mutant and wild-type MB-COMTs (data not shown). The sizes of the expressed proteins matched the expected sizes for the recombinant human S- and MB-COMT proteins.

Kinetic parameters for the O-methylation of 2-OH-E₂ and 4-OH-E₂. To characterize the kinetic parameters (K_M , V_{MAX}

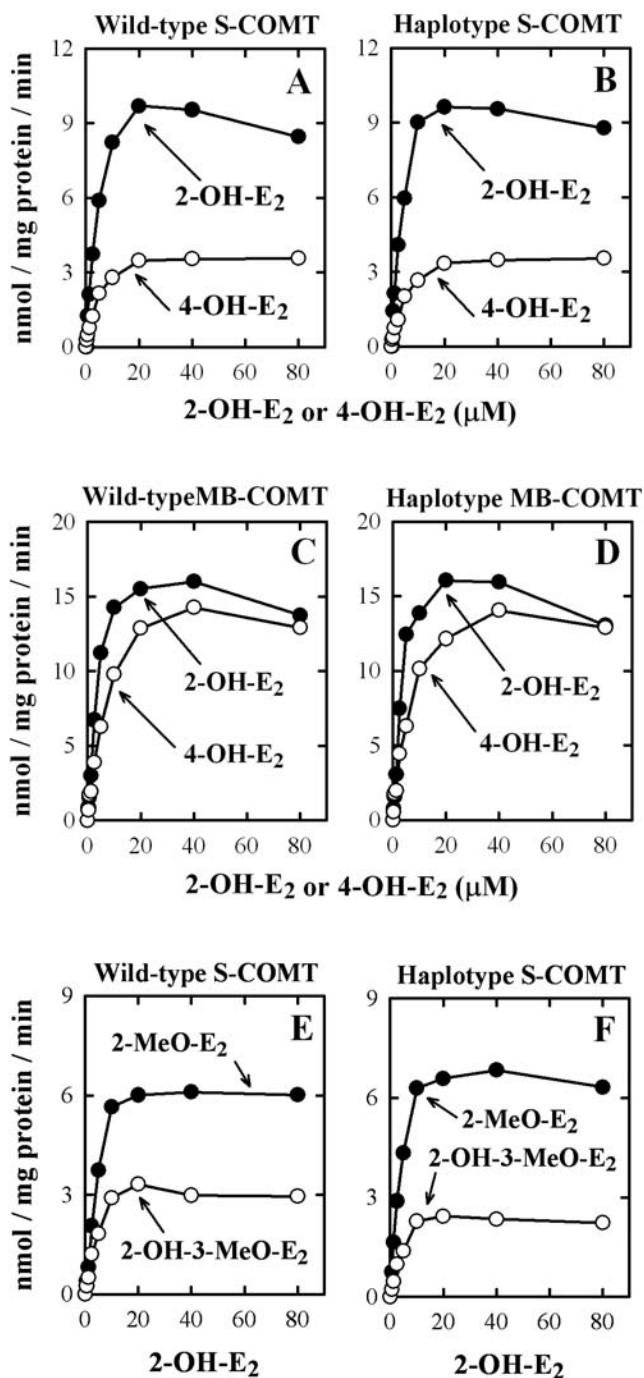


Figure 3. Relationship between catechol estrogen concentrations and their rate of *O*-methylation by wild-type human COMTs (A, C and E) and the haplotype COMTs (B, D, and F). Panel E shows the rate of 2-*O*-methylation and 3-*O*-methylation of 2-OH-E₂ catalyzed by the wild-type S-COMT, and panel F shows the rate of 2-*O*-methylation and 3-*O*-methylation of 2-OH-E₂ by mutant S-COMT. The incubation mixture consisted of 10 different concentrations (0, 0.31, 0.63, 1.25, 2.5, 5, 10, 20, 40, and 80 μM) of each substrate, 1.2 mM MgCl₂, 100 μM AdoMet (containing 0.5 mCi [methyl-³H]AdoMet), 1 mM 1,4-dithiothreitol, and the recombinant COMT protein (at 16.2 μg/ml for S-COMT or 17.1 μg/ml for MB-COMT). The incubations were carried out at 37°C for 15 min. Note that the rate of its total methylation was based on the liquid scintillation counting of the radioactivity extracted with ethyl acetate. The rates for its 2-*O*- and 3-*O*-methylation (C and F) were determined by using HPLC that separately quantified the amount of 2-methoxyestradiol (2-MeO-E₂) and 2-OH-E₂ 3-methyl ether (2-OH-3-MeO-E₂) formed. Each value is the mean of duplicate measurements.

and V_{MAX}/K_M) of the mutant human S- and MB-COMTs, we selectively expressed them and also the wild-type S- and MB-COMTs in *E. coli*. The enzyme activity assays were performed by measuring the COMT-mediated *O*-methylation of 2-OH-E₂ and 4-OH-E₂, two representative endogenous catechol estrogen substrates. No significant difference was observed between the wild-type S-COMT and its D51G/S60F/K162R mutant form, or between the wild-type MB-COMT and its D101G/S110F/K212R mutant form (Fig. 2 and Table II).

When a fixed concentration of 2-OH-E₂ or 4-OH-E₂ (at 10 μM) was used as a substrate, the apparent K_M values of the wild-type S-COMT for AdoMet were 113.6 or 145.3 μM, respectively (Fig. 3). Under the same conditions, the apparent K_M values of the D51G/S60F/K162R mutant of human S-COMT for AdoMet were 79.5 and 146.7 μM. Notably, the K_M value of the mutant S-COMT for AdoMet in the *O*-methylation of 2-OH-E₂ (but not 4-OH-E₂) was ~50% lower than the corresponding K_M value for the wild-type S-COMT (Table II).

We have also compared the sensitivity of the mutant and wild-type human S-COMTs to inhibition by AdoHcy (a physiological feedback inhibitor of COMTs) on the *O*-methylation of 2-OH-E₂ and 4-OH-E₂. The mutant D51G/S60F/K162R S-COMT was less sensitive to inhibition by AdoHcy for the *O*-methylation of 2-OH-E₂ as compared to the wild-type S-COMT (IC_{50} values of 18.4 and 6.07 μM, respectively, Fig. 4). However, AdoHcy had a comparable potency for inhibiting the *O*-methylation of 4-OH-E₂ by the wild-type S-COMT and its D51G/S60F/K162R mutant (the IC_{50} values of 22.6 and 29.6 μM, respectively) (Fig. 4).

Thermostability test. The thermostability of human COMTs has often been used as an indicator of the biophysical properties of the mutant COMT proteins. In the present study, we also compared the thermostability of the mutant human S- and MB-COMTs with the wild-type COMTs. The D51G/S60F/K162R mutant S-COMT and the D101G/S110F/K212R mutant MB-COMT were more sensitive to inactivation after pre-incubation at 37°C. The mutant S-COMT and MB-COMT lost ~60% of their catalytic activity after pre-incubation at 37°C for 120 min, while the wild-type enzymes were mostly stable after up to 120 min of pre-incubation at 37°C (Fig. 5).

Notably, the thermostability of the S60F/K162R mutant S-COMT, which has one of the three point mutations corrected by site-directed mutagenesis, was completely restored to that of the wild-type COMT (data not shown). These data showed that the aspartate residue at position 51 (D51) of the human S-COMT plays a crucial role in determining its thermostability.

Molecular modeling. To better understand the mechanism of the triplet point mutations in affecting the structure and catalytic activity of human COMTs, we constructed the homology models for wild-type and mutant human S-COMTs. Two different methods were used to build the mutant human S-COMT. One of them used the human S-COMT homology model as a template to build the structure

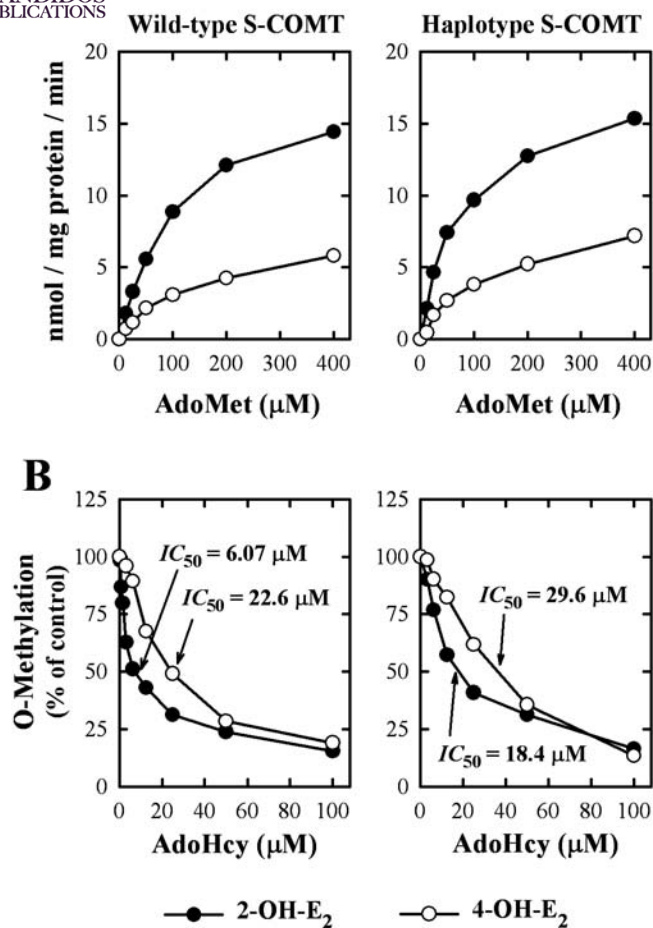


Figure 4. Biochemical properties of the *in vitro* O-methylation of catechol substrates with respect to AdoMet and AdoHcy concentrations. The representative substrates used in these assays were 2-OH-E₂ and 4-OH-E₂ at 10 μM concentrations. The incubation mixture consisted of the substrate, 100 μM [methyl-³H]AdoMet (containing 0.2 μCi or as indicated), 16.2 $\mu\text{g}/\text{ml}$ of wild-type S-COMT or haplotype S-COMT, 1 mM 1,4-dithiothreitol, and 1.2 mM MgCl₂ in a final volume of 300 μl Tris-HCl buffer (50 mM) at pH 7.4. The incubations were carried out at 37°C for 15 min. Each value is the mean of duplicate measurements.

of the mutant S-COMT. This method has been commonly used in many other similar studies. In addition, we also built *de novo* the structure of the mutant human S-COMT according to the known crystallographic structure of the rat S-COMT. The use of two different methods was hoped to provide a more complete view of the possible structures of this new mutant human COMT. Our homology models showed that the two mutant S-COMT homology models built with two different templates are nearly the same in their backbone structures as well as their catalytic sites (Fig. 6), with only minor differences in the side-chain orientations. Therefore, for further structural comparison with the wild-type human S-COMT as well as for docking calculations using representative substrates, the mutant S-COMT built according to the wild-type human S-COMT was used.

When the enzyme was complexed with a representative substrate and also the methyl donor AdoMet, the secondary structures of the wild-type and mutant S-COMTs were nearly identical (Fig. 6). Although the S60F mutation caused some minor changes in loop 5 which connects α -helix 4 and β -

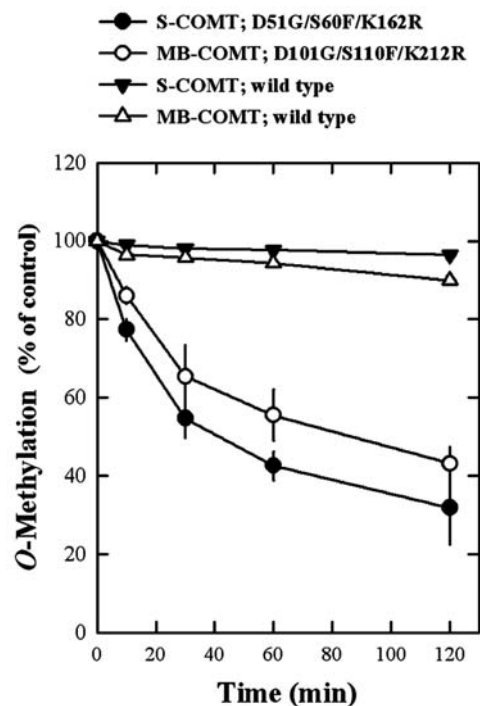


Figure 5. Stabilities of the wild-type and haplotype human recombinant S- and MB-COMTs. The reaction mixtures consisted of the recombinant COMT protein (at 16.2 $\mu\text{g}/\text{ml}$ for S-COMT or 17.1 $\mu\text{g}/\text{ml}$ for MB-COMT), 1.2 mM MgCl₂, 100 μM AdoMet (containing 0.5-1 mCi [methyl-³H]AdoMet), 2-OH-E₂ as a substrate and 1 mM 1,4-dithiothreitol in Tris-HCl buffer (50 mM, pH 7.4). The final volume of the reaction mixture was usually 300 μl . The reaction was initiated by the addition of the recombinant human COMT protein and carried out at 37°C for 15 min. To test the thermostability of the mutant and wild-type COMTs, the enzymes were first preincubated at 37°C for the indicated length of time immediately before testing their catalytic activity for the O-methylation of 2-OH-E₂ (at 10 μM). Each value is the mean \pm S.D. of triplicate measurements.

sheet 2, the overall structure and configuration of the nine α -helices and seven β -sheets remained mostly the same. The amino acid residue D51 in the wild-type S-COMT was located in α -helix 3, and S60 and K162 were located in two different loop regions (loops 3 and 11, respectively). A comparison of homology-modeled structures of the wild-type and mutant form showed that the D51G mutation did not noticeably interrupt the structure of α -helix 3 of the enzyme, and similarly, the S60K and K162R mutations also did not significantly affect the overall structure of the enzyme. Notably, these three mutations are mostly located in the outer surface regions of the COMT protein, and they are on the opposite side of the catalytic pocket. The distances between the α -carbon of D51, S60 and K162 to Mg²⁺ are 13.8, 23.2 and 25.2 \AA , respectively, which are beyond the defined catalytic site (within the 7- \AA reach of the Mg²⁺ ion). The structures of the catalytic sites in the wild-type and mutant COMTs are nearly identical when they are in complex with 2-OH-E₂ or 4-OH-E₂. In addition, the orientations of the two substrates are very similar when they are bound as a substrate in the catalytic pocket.

Using the homology models we developed, we also computed the relative binding energy values ($\Delta E_{\text{binding}}$) of the wild-type and mutant human COMTs for 2-OH-E₂ and 4-OH-E₂. We found that 2-OH-E₂ and 4-OH-E₂ have similar

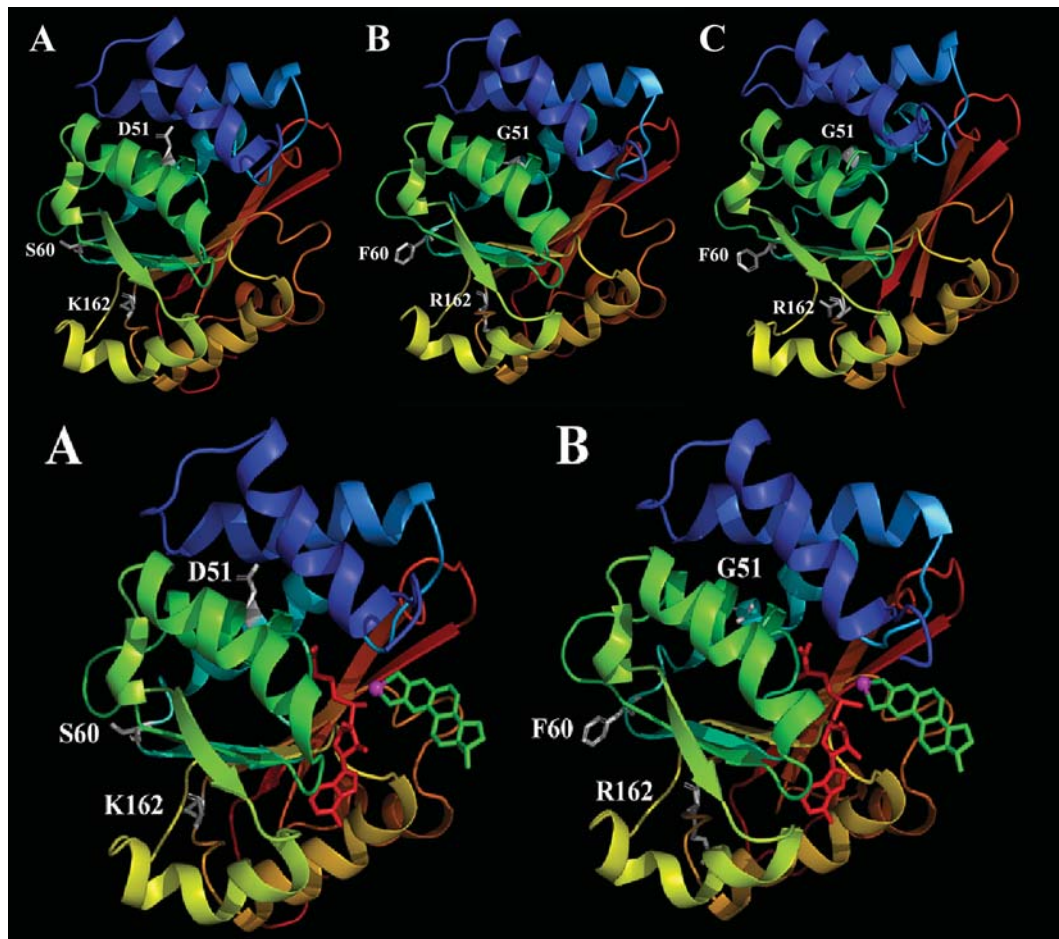


Figure 6. Computational molecular modeling analysis of the wild-type and new haplotype human COMT structures. Upper panel. The homology models of the wild-type human S-COMT (A), the D51G/S60F/K162R mutant S-COMT built according to the homology model of the wild-type human S-COMT (B), or according to the crystallographic structure of the rat S-COMT (PDB code: 1VID) (C). Note that AdoMet, Mg^{2+} and substrates are not included in the development of this initial homology model. The figure is drawn with the PyMOL software. Secondary structures are shown with colored ribbons with blue for *N*-terminus and red for *C*-terminus. The amino acids at mutation sites (D51, S60 and K162 for wild-type S-COMT and G51, F60 and R162 for mutant S-COMT) are shown in white sticks. Hydrogens are omitted from the amino acids. Lower panel. The homology models of the wild-type human S-COMT (A) and the D51G/S60F/K162R mutant S-COMT (built according to the homology model of the wild-type human S-COMT) (B) in the presence of AdoMet, Mg^{2+} and a representative substrate (2-OH- E_2). The enzymes are complexed with 2-OH- E_2 in the catalytic pocket in its geometry for 2-*O*-methylation. The figure is drawn using PyMOL software. Secondary structures are shown with colored ribbons with blue for *N*-terminus and red for *C*-terminus. AdoMet is colored red, 2-OH- E_2 , green and Mg^{2+} , magenta. The amino acids at mutation sites (D51, S60 and K162 for wild-type S-COMT and G51, F60 and R162 for mutant S-COMT) are shown in white sticks. Hydrogens are omitted from AdoMet, 2-OH- E_2 and the amino acids.

binding energy values for the wild-type and mutant S-COMTs (data summarized in Table III). When 2-OH- E_2 was the substrate, the $\Delta E_{\text{binding}}$ values for its interactions with the wild-type and mutant S-COMTs for its 2-*O*-methylation were relatively comparable, -124.5 and -135.4 kcal/mol, respectively (Table III), suggesting that the wild-type and mutant S-COMTs have a similar binding affinity for 2-OH- E_2 , which agreed well with our experimental data (Table II). Similarly, the computed binding energy values for the binding interactions of 4-OH- E_2 with the wild-type and mutant S-COMTs for its 4-*O*-methylation were -122.9 and -138.5 kcal/mol, respectively (Table III), which are also in agreement with our experimental data (Table II).

Discussion

Earlier familial studies based on the measurement of COMT activity in red blood cells revealed that COMT is a poly-

morphic enzyme, with a bimodal/trimodal distribution pattern (39). Moreover, the trait of the low COMT activity was found to be associated with a decreased thermostability of the enzyme preparation (22-24). Following the subsequent cloning of the human *COMT* gene (4,40,41), a number of single point mutations in the coding regions of this gene have been reported (listed in Table I). Among them, the V108/158M polymorphism has been most extensively studied. Many epidemiological studies have shown that women, homozygous with the V108/158M mutant, have an increased risk of developing estrogen-associated cancers (21-27). This polymorphism has also been suggested to be associated with an elevated risk of schizophrenia, obsessive-compulsive disorder, bipolar disorder, and Parkinson's disease in both genders (22-24,28-32). In addition to the known effects associated with non-synonymous mutations, polymorphism involving synonymous changes (such as H62H and L136L) has also been shown to cause a marked change in COMT



Genotype	Substrate	Site of <i>O</i> -methylation	$\Delta E_{\text{binding}}$
Wild-type	2-OH-E ₂	2- <i>O</i> -methylation	-124.5
		3- <i>O</i> -methylation	-121.9
	4-OH-E ₂	3- <i>O</i> -methylation	-122.9
		4- <i>O</i> -methylation	-123.2
Mutant (D51G/S60F/K162R)	2-OH-E ₂	2- <i>O</i> -methylation	-135.4
		3- <i>O</i> -methylation	-148.7
	4-OH-E ₂	3- <i>O</i> -methylation	-138.5
		4- <i>O</i> -methylation	-143.4

$\Delta E_{\text{binding}}$ was calculated using the following equation: $\Delta E_{\text{binding}} = E_{\text{complex}} - (E_{\text{COMT}} + E_{\text{substrate}})$, where E_{complex} is the potential energy for the complex of COMT with substrate, E_{COMT} is the potential energy of the enzyme itself and $E_{\text{substrate}}$ is the potential energy for the substrate itself.

levels, largely due to reduced amounts of the COMT proteins being expressed (12). These studies provide the basis for further studies to identify other non-synonymous and synonymous mutations/polymorphisms of the human *COMT* gene and particularly those that alter the catalytic activity of COMT and/or its protein levels.

In the present study, we identified a new haplotype of the human *COMT* gene with triplet point mutations (D51G/S60F/K162R for S-COMT and D101G/S110F/K212R for MB-COMT), and we have also selectively expressed the mutant enzymes in *E. coli* for the characterization of the catalytic properties. We found that the kinetic parameters (K_M and V_{MAX}) of the mutant S- and MB-COMTs did not differ significantly from those of the wild-type enzymes when 2-OH-E₂ and 4-OH-E₂ were used as substrates. For instance, the mutant S- and MB-COMTs have similar K_M values (3.3 and 2.6 μM , respectively) as the wild-type S- and MB-COMTs (3.6 and 3.2 μM , respectively) for the *O*-methylation of 2-OH-E₂. In addition, the mutant S-COMT had almost the same regio-preference for the 2-*O*-methylation of 2-OH-E₂ over its 3-*O*-methylation as that of the wild-type S-COMT.

One of the notable differences between the mutant and wild-type human COMTs was the thermostability. While the wild-type human COMTs (S- and MB-COMT) are very stable after 2 h of pre-incubation at 37°C, the mutant COMTs lost ~60% of their catalytic activity under the same pre-incubation conditions. Notably, earlier biochemical analyses of the V108M mutant and wild-type human S-COMTs also showed that whereas they had similar catalytic activity for the *O*-methylation of catechol estrogens *in vitro*, the mutant protein was more susceptible to heat inactivation (35,36). In these cases, since the mutant enzymes are more unstable under prolonged incubation at 37°C (the physiological temperature), it is suggested that this will eventually result in decreased total levels of catalytically-active COMTs in a given tissue or cell.

In addition, we also noted a difference in the apparent K_M values of the mutant and wild-type S-COMTs for the methyl

donor AdoMet. Whereas the mutant S-COMT had a lower K_M value than did the wild-type S-COMT when 2-OH-E₂ was used as substrate, this difference disappeared when 4-OH-E₂ was the substrate. As expected, the mutant and wild-type S-COMTs also had a different sensitivity to inhibition by AdoHcy, a demethylated AdoMet which binds to same pocket in the COMTs. The IC_{50} value (18.4 μM) of AdoHcy for the mutant S-COMT was 3-fold higher than the IC_{50} value (6.1 μM) for the wild-type S-COMT when 2-OH-E₂ was the substrate, but when 4-OH-E₂ was the substrate, the sensitivity of inhibition by AdoHcy was comparable (26.6 and 29.6 μM). Collectively, these data suggest that the mutant S-COMT has a higher binding affinity for AdoMet than does the wild-type S-COMT when 2-OH-E₂ is the substrate, and due to its higher binding affinity, the concentration of AdoHcy needed to compete for its binding site is proportionally higher.

To better understand the precise structural and functional differences between the D51G/S60F/K162R mutant of S-COMT and the wild-type protein, we have built the homology models of the mutant and wild-type S-COMTs for comparison. It is apparent that the overall secondary structure of the mutant enzyme and especially the structure of its catalytic site (built in two different ways) are very similar to those of the wild-type enzyme. Consistent with this observation, the calculated binding energy values ($\Delta E_{\text{binding}}$) for the interactions of the mutant and wild-type enzymes with substrates were also comparable, which agreed well with the experimental data for the *O*-methylation of 2-OH-E₂ and 4-OH-E₂. The overall structural similarities between the mutant and wild-type S-COMTs likely are attributable to the following two factors: i) The three mutant amino acid residues were found to be located at the surface regions of the protein and were relatively distanced from the catalytic site. ii) Two of the three mutations were located in the loop regions of the protein sequence (S60F in loop 3 and K162R in loop 11), and their presence did not alter any of the existing α -helical structures. Although D51 is located in the middle of α -helix 3, this mutation also did not cause a disruption of the original α -helical structure.

In summary, we have identified a new human *COMT* haplotype with triple point mutations, i.e., D51G/S60F/K162R for S-COMT and D101G/S110F/K212R for MB-COMT. The selectively-expressed S- and MB-COMT proteins have been biochemically characterized for their catalytic and kinetic properties. Although the catalytic activity of the mutant S- and MB-COMTs does not differ significantly from that of the wild-type enzymes when 2-OH-E₂ or 4-OH-E₂ is the substrate, the mutant COMTs have a lower thermostability compared to the wild-type proteins. The mutant and wild-type S-COMTs have different binding affinities for AdoMet and AdoHcy, depending on the substrate used.

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References

- Axelrod J and Tomchick R: Enzymatic *O*-methylation of epinephrine and other catechols. *J Biol Chem* 233: 702-705, 1958.
- Axelrod J: Methylation reactions in the formation and metabolism of catecholamines and other biogenic amines. *Pharmacol Rev* 18: 95-113, 1966.
- Zhu BT: Catechol-*O*-methyltransferase (COMT)-mediated methylation metabolism of endogenous bioactive catechols and modulation by endobiotics and xenobiotics: importance in pathophysiology and pathogenesis. *Curr Drug Metab* 3: 321-49, 2002.
- Tenhunen J, Salminen M, Jalanko A, Ukkonen S and Ulmanen I: Structure of the rat catechol-*O*-methyltransferase gene: separate promoters are used to produce mRNAs for soluble and membrane-bound forms of the enzyme. *DNA Cell Biol* 12: 253-263, 1993.
- Zhu BT and Liehr JG: Quercetin increases the severity of estradiol-induced tumorigenesis in hamster kidney. *Toxicol Appl Pharmacol* 125: 149-158, 1994.
- Zhu BT, Patel UK, Cai MX and Conney AH: *O*-methylation of tea polyphenols catalyzed by human placental cytosolic catechol-*O*-methyltransferase. *Drug Metab Dispos* 28: 1024-1030, 2000.
- Zhu BT, Ezell EL and Liehr JG: Catechol-*O*-methyltransferase-catalyzed rapid *O*-methylation of mutagenic flavonoids. Metabolic inactivation as a possible reason for their lack of carcinogenicity in vivo. *J Biol Chem* 269: 292-299, 1994.
- Zhu BT and Liehr JG: Inhibition of catechol *O*-methyltransferase-catalyzed *O*-methylation of 2- and 4-hydroxyestradiol by quercetin. Possible role in estradiol-induced tumorigenesis. *J Biol Chem* 271: 1357-1363, 1996.
- Zhu BT: CNS dopamine oxidation and catechol-*O*-methyltransferase: importance in the etiology, pharmacotherapy, and dietary prevention of Parkinson's disease. *Int J Mol Med* 13: 343-353, 2004.
- Nackley AG, Shabalina SA, Tchivileva IE, Satterfield K, Korchynskiy O, Makarov SS, Maixner W and Diatchenko L: Human catechol-*O*-methyltransferase haplotypes modulate protein expression by altering mRNA secondary structure. *Science* 314: 1930-1933, 2006.
- Diatchenko L, Slade GD, Nackley AG, Bhalang K, Sigurdsson A, Belfer I, Goldman D, Xu K, Shabalina SA, Shagin D, Max MB, Makarov SS and Maixner W: Genetic basis for individual variations in pain perception and the development of a chronic pain condition. *Hum Mol Genet* 14: 135-143, 2005.
- Shield AJ, Thomae BA, Eckloff BW, Wieben ED and Weinshilboum RM: Human catechol *O*-methyltransferase genetic variation: gene resequencing and functional characterization of variant allozymes. *Mol Psychiatry* 9: 151-160, 2004.
- Karayorgou M, Altemus M, Galke BL, Goldman D, Murphy DL, Ott J and Gogos JA: Genotype determining low catechol-*O*-methyltransferase activity as a risk factor for obsessive-compulsive disorder. *Proc Natl Acad Sci USA* 94: 4572-4575, 1997.
- Kirov G, Murphy KC, Arranz MJ, Jones I, McCandles F, Kunugi H, Murray RM, McGuffin P, Collier DA, Owen MJ and Craddock N: Low activity allele of catechol-*O*-methyltransferase gene associated with rapid cycling bipolar disorder. *Mol Psychiatry* 3: 342-345, 1998.
- Papoulos DF, Veit S, Faedda GL, Saito T and Lachman HM: Ultra-ultra rapid cycling bipolar disorder is associated with the low activity catecholamine-*O*-methyltransferase allele. *Mol Psychiatry* 3: 346-349, 1998.
- Strous RD, Bark N, Parsia SS, Volavka J and Lachman HM: Analysis of a functional catechol-*O*-methyltransferase gene polymorphism in schizophrenia: evidence for association with aggressive and antisocial behavior. *Psychiatry Res* 69: 71-77, 1997.
- Tiihonen J, Hallikainen T, Lachman H, Saito T, Volavka J, Kauhanen J, Syvälahti E, Hietala J and Tiihonen J: Association between the functional variant of the catechol-*O*-methyltransferase (COMT) gene and type 1 alcoholism. *Mol Psychiatry* 4: 286-289, 1999.
- Enoch MA, Xu K, Ferro E, Harris CR and Goldman D: Genetic origins of anxiety in women: a role for a functional catechol-*O*-methyltransferase polymorphism. *Psychiatr Genet* 13: 33-41, 2003.
- Rosa A, Peralta V, Cuesta MJ, Zarzuela A, Serrano F, Martinez-Larrea A and Fañanás L: New evidence of association between COMT gene and prefrontal neurocognitive function in healthy individuals from sibling pairs discordant for psychosis. *Am J Psychiatry* 161: 1110-1112, 2004.
- Nolan KA, Bilder RM, Lachman HM and Volavka J: Catechol *O*-methyltransferase Val158Met polymorphism in schizophrenia: differential effects of Val and Met alleles on cognitive stability and flexibility. *Am J Psychiatry* 161: 359-361, 2004.
- Illi A, Mattila KM, Kampman O, Anttila S, Roivas M and Lehtimäki T: Catechol-*O*-methyltransferase and monoamine oxidase A genotypes and drug response to conventional neuroleptics in schizophrenia. *J Clin Psychopharmacol* 23: 429-434, 2003.
- Zhu BT and Conney AH: Functional role of estrogen metabolism in target cells: review and perspectives. *Carcinogenesis* 19: 1-27, 1998.
- Lee AJ, Cai MX, Tomas PE, Conney AH and Zhu BT: Characterization of the oxidative metabolites of 17 β -estradiol and estrone formed by 15 selectively expressed human cytochrome p450 isoforms. *Endocrinology* 144: 3382-3398, 2003.
- Zhu BT and Conney AH: Is 2-methoxyestradiol an endogenous estrogen metabolite that inhibits mammary carcinogenesis? *Cancer Res* 58: 2269-2277, 1998.
- Huang CS, Chern HD, Chang KJ, Cheng CW, Hsu SM and Shen CY: Breast cancer risk associated with genotype polymorphism of the estrogen-metabolizing genes CYP17, CYP1A1, and COMT: A multigenic study on cancer susceptibility. *Cancer Res* 59: 4870-4875, 1999.
- Mitrunen K, Jourenkova N, Kataja V, Eskelinen M, Kosma VM, Benhamou S, Vainio H, Uusitupa M and Hirvonen A: Glutathione S-transferase M1, M3, P1, and T1 genetic polymorphisms and susceptibility to breast cancer. *Cancer Epidemiol Biomarkers Prev* 10: 635-640, 2001.
- Mitrunen K, Kataja V, Eskelinen M, Kosma VM, Kang D, Benhamou S, Vainio H, Uusitupa M and Hirvonen A: Combined COMT and GST genotypes and hormone replacement therapy associated breast cancer risk. *Pharmacogenetics* 12: 67-72, 2002.
- Thompson PA, Shields PG, Freudenheim JL, Stone A, Vena JE, Marshall JR, Graham S, Laughlin R, Nemoto T, Kadlubar FF and Ambrosone CB: Genetic polymorphisms in catechol-*O*-methyltransferase, menopausal status, and breast cancer risk. *Cancer Res* 58: 2107-2110, 1998.
- Suzuki K, Nakazato H, Matsui H, Koike H, Okugi H, Kashiwagi B, Nishii M, Ohtake N, Nakata S, Ito K and Yamanaka H: Genetic polymorphisms of estrogen receptor alpha, CYP19, catechol-*O*-methyltransferase are associated with familial prostate carcinoma risk in a Japanese population. *Cancer* 98: 1411-1416, 2003.
- Yim DS, Parkb SK, Yoo KY, Yoon KS, Chung HH, Kang HL, Ahn SH, Noh DY, Choe KJ, Jang IJ, Shin SG, Strickland PT, Hirvonen A and Kang D: Relationship between the Val158Met polymorphism of catechol *O*-methyl transferase and breast cancer. *Pharmacogenetics* 11: 279-286, 2001.



SPANDIDOSian MT, McDuffie K, Kolonel LN, Terada K, Donlon TA,
PUBLICATIONSis LR, Guo C and Le Marchand L: Case-control study of

- ovarian cancer and polymorphisms in genes involved in catecholesterogen formation and metabolism. *Cancer Epidemiol Biomarkers Prev* 10: 209-216, 2001.
32. Lavigne JA, Helzlsouer KJ, Huang HY, Strickland PT, Bell DA, Selmin O, Watson MA, Hoffman S, Comstock GW and Yager JD: An association between the allele coding for a low activity variant of catechol-*O*-methyltransferase and the risk for breast cancer. *Cancer Res* 57: 5493-5497, 1997.
33. Lee SG, Joo Y, Kim B, Chung S, Kim HL, Lee I, Choi B, Kim C and Song K: Association of Ala72Ser polymorphism with COMT enzyme activity and the risk of schizophrenia in Koreans. *Hum Genet* 116: 319-328, 2005.
34. Lotta T, Vidgren J, Tilgman C, Ulmanen I, Melen K, Julkunen I and Taskinen J: Kinetics of human soluble and membrane-bound catechol *O*-methyltransferase: a revised mechanism and description of the therolabile variant of the enzyme. *Biochemistry* 34: 4202-4210, 1995.
35. Li Y, Yao J, Chang M, Nikolic D, Yu L and Yager JD: Equine catechol estrogen 4-hydroxyequilenin is a more potent inhibitor of the variant form of catechol *O*-methyltransferase. *Chem Res Toxicol* 17: 512-520, 2004.
36. Goodman JE, Jensen LT, He P and Yager JD: Characterization of human soluble high and low activity catechol *O*-methyltransferase catalyzed catechol estrogen methylation. *Pharmacogenetics* 12: 517-528, 2002.
37. Li Y, Yang X, Breemen RB and Bolton JL: Characterization of two new variants of human catechol *O*-methyltransferase *in vitro*. *Cancer Lett* 230: 81-89, 2005.
38. Zhu BT, Roy D and Liehr JG: The carcinogenic activity of ethinyl estrogens is determined by both their hormonal characteristics and their conversion to catechol metabolites. *Endocrinology* 132: 577-583, 1993.
39. Cohn CC, Dunner DL and Axelrod J: Reduced catechol-*O*-methyltransferase activity in red blood cells of women with primary affective disorder. *Science* 170: 125-135, 1970.
40. Tenhunen J and Ulmanen I: Production of rat soluble and membrane-bound catechol *O*-methyltransferase forms from bifunctional mRNAs. *Biochem J* 296(Pt 3): 595-600, 1993.
41. Winqvist R, Lundstrom K, Salminen M, Laatikainen M and Ulmanen I: The human catechol-*O*-methyltransferase (COMT) gene maps to band q11.2 of chromosome 22 and shows a frequent RFLP with BglI. *Cytogenet Cell Genet* 59: 253-257, 1992.