Published in final edited form as:

ACS Chem Biol. 2017 February 17; 12(2): 342–346. doi:10.1021/acscchembio.6b00922.

Kinetic Isotope Effects and Transition State Structure for Human Phenylethanolamine *N*-Methyltransferase

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Abstract

Phenylethanolamine *N*-methyltransferase (PNMT) catalyzes the *S*-adenosyl-L-methionine (SAM)-dependent conversion of norepinephrine to epinephrine. Epinephrine has been associated with critical processes in humans including the control of respiration and blood pressure. Additionally, PNMT activity has been suggested to play a role in hypertension and Alzheimer's disease. In the current study, labeled SAM substrates were used to measure primary *methyl*-¹⁴C and ³⁶S and secondary *methyl*-³H, 5'-³H, and 5'-¹⁴C intrinsic kinetic isotope effects for human PNMT. The transition state of human PNMT was modeled by matching kinetic isotope effects predicted *via* quantum chemical calculations to intrinsic values. The model provides information on the geometry and electrostatics of the human PNMT transition state structure and indicates that human PNMT catalyzes the formation of epinephrine through an early S_N2 transition state in which methyl transfer is rate-limiting.

Graphical abstract

Phenylethanolamine *N*-methyltransferase (PNMT) catalyzes the *S*-adenosyl-L-methionine (SAM)-dependent conversion of norepinephrine to epinephrine (Figure 1a) in the

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ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acscchembio.6b00922.

Figures S1–S5 and Tables S1 and S2, complete experimental procedures for expression and purification of wild-type hPNMT, synthesis of isotopically labeled SAM substrates, measurement of *V*/*K* KIEs for hPNMT, measurement of forward commitment for hPNMT, and computational methods (PDF)

The authors declare no competing financial interest.

catecholamine biosynthetic pathway. Expression of PNMT in humans (hPNMT) occurs largely in the adrenal gland where epinephrine functions as a hormone.¹ However, hPNMT is also expressed in several other organs including the heart, retina, and brain.^{2,3} Though the role of hPNMT in the human central nervous system (CNS) has yet to be fully elucidated, epinephrine has been associated with a number of CNS processes such as cardiovascular homeostasis, adrenergic receptor activation, learning and memory, and regulation of the circadian cycle.¹ In addition, studies suggest hPNMT may play a role in several human disease states including hypertension,⁴ myocardial infarction,⁵ Alzheimer's disease,⁶ and Parkinson's disease.^{7,8}

Recently, density functional theory (DFT)⁹ and quantum mechanics/molecular mechanics (QM/MM)¹⁰ methods have been used to investigate the hPNMT reaction mechanism. These studies suggest that hPNMT catalyzes the formation of epinephrine through an S_N2 transition state (TS) in which the methyl transfer step is rate-limiting. Importantly, this computational proposal can be experimentally tested by kinetic isotope effect (KIE) analysis of the hPNMT reaction. KIEs report on bond vibrational changes between the ground state (GS) and TS of a chemical reaction. As such, experimental KIEs can serve as boundary constraints for quantum chemical calculations to model reactant structure at the TS.^{11,12} Information provided by such analyses is critical in the design of TS analogs,^{11,12} which have been shown to function as extremely potent enzyme inhibitors.¹³ Previously, our lab reported TS structures for the human protein lysine methyltransferase NSD2¹⁴ and human DNA methyltransferase 1 (DNMT1).¹⁵ In the current study, we extended these analyses to hPNMT by measuring intrinsic KIEs and generating a model for the TS structure of the methyl transfer reaction. Our model provides subangstrom detail of the hPNMT TS and supports methyl transfer as the rate-limiting step of an S_N2 process.

KIEs on $V_{\max}/K_M$ (V/K) for the hPNMT reaction were measured *via* the competitive radiolabel method,^{11,12} as previously reported for NSD2¹⁴ and DNMT1.¹⁵ In this approach, SAM substrates selectively labeled with radioisotopes at either sensitive ("heavy") or remote positions ("light") are mixed and compete for the enzyme active site (Figure 1b and Table 1). Accordingly, V/K KIEs report on all steps of the reaction up to and including the first irreversible chemical step.¹⁶ Previous studies on hPNMT indicate that methyl transfer proceeds irreversibly, with both epinephrine and *S*-adenosyl-L-homocysteine (SAH) functioning as competitive inhibitors.^{17–19} As the kinetic mechanism of hPNMT is an ordered process with SAM binding first,¹⁹ V/K KIEs may not accurately reflect the intrinsic values if SAM exhibits forward commitment (C_f). Intrinsic KIEs (k^*), which report on the chemical step alone, can be extracted from V/K values if C_f is known, as illustrated by eq 1.¹⁶

$$V/K = (k^* + C_f) / (1 + C_f) \quad (1)$$

The substrate trapping method²⁰ was used to measure C_f for SAM with hPNMT, which was determined to be 0.074 ± 0.001 (Supporting Information Figure S1) under conditions identical to those used in the KIE measurements. Intrinsic KIEs were calculated from the V/K values using eq 1 (Table 1).

Primary *methyl*- ^{14}C and ^{36}S KIEs on the hPNMT reaction were determined to be 1.116 ± 0.005 and 1.014 ± 0.005 , respectively (Table 1). This primary *methyl*- ^{14}C KIE is consistent with values reported for NSD2,¹⁴ DNMT1,¹⁵ and glycine *N*-methyltransferase (GNMT).²¹ The large magnitude of this KIE indicates that methyl transfer for hPNMT proceeds *via* a rate-limiting $\text{S}_{\text{N}}2$ mechanism.²² These isotope effects are in agreement with previous studies that support methyl transfer as the rate-limiting step of the PNMT reaction.^{9,10} DFT calculations for NSD2¹⁴ and DNMT1¹⁵ suggest that the primary ^{36}S KIE on methyltransferase reactions is proportional to the loss of bond order between the carbon of the transferring methyl (C_{Me}) and the sulfur of SAM at the TS. The ^{36}S KIE for hPNMT (1.014 ± 0.005) is smaller than those measured for NSD2 (1.018 ± 0.008)¹⁴ and DNMT1 (1.019 ± 0.006),¹⁵ suggesting that hPNMT TS structure may retain somewhat greater $\text{C}_{\text{Me}}\text{--S}$ bond order. This observation also suggests that the TS of hPNMT is early relative to those of NSD2 and DNMT1, though the ^{36}S KIEs for these three enzymes are similar within experimental error. The α -secondary *methyl*- ^3H KIE was measured as 0.796 ± 0.006 for hPNMT (Table 1). The inverse value of this KIE indicates increased force constants on the $\text{C}_{\text{Me}}\text{--H}$ bonds at the TS, which is consistent with the corresponding α -secondary KIEs measured for NSD2,¹⁴ GNMT,²¹ and catechol *O*-methyl-transferase (COMT).^{23–25} Finally, the α -secondary $5'\text{--}^{14}\text{C}$ and β -secondary $5'\text{--}^3\text{H}$ KIEs for hPNMT were determined to be 0.996 ± 0.009 and 1.047 ± 0.006 , respectively. Previous studies on the NSD2¹⁴ and DNMT¹⁵ indicate these KIEs are influenced by changes in the geometry of SAM upon protein binding, as well as interactions between the substrate and protein residues at the TS.

To further define the hPNMT mechanism, we used the intrinsic KIEs as constraints for TS structures calculated using DFT methods. Geometries of the TS structures were refined such that predicted KIEs matched the corresponding intrinsic values within experimental error (Table 1). KIEs were calculated (ISOEFF98)²⁷ from the scaled vibrational frequencies of optimized structures (Gaussian 09, RB3LYP/6-31g(d) theory)²⁶ of SAM in the GS and at the TS. The input geometry for the GS structure of SAM was taken from the coordinates of the low energy conformer in the PubChem database (CID: 24762165).²⁸ The full SAM structure was truncated to a diethyl(methyl)sulfonium species, which was then optimized using water as an implicit solvent (polarizable continuum model, PCM). The energy minimization step did not alter the geometry of the simplified model relative to the fully elaborated SAM conformer (Supporting Information Figure S2). This diethyl(methyl)sulfonium model of SAM was employed in previous DFT studies on the hPNMT TS and is regarded as sufficient to reproduce the reactivity of the $\text{C}_{\text{Me}}\text{--S}$ bond.⁹ The $5'\text{--}^3\text{H}_2$ and $5'\text{--}^{14}\text{C}$ KIEs were calculated for the *pro-S* methylene group of the diethyl(methyl)sulfonium model, as this position corresponds to the $5'$ carbon in the full SAM structure (Supporting Information Figure S2). Input geometries for the TS search were taken from the crystal structure of the hPNMT·norepinephrine·SAH ternary complex (PDB: 3HCD).²⁹ Overlaying the bound structure of SAH with that of SAM in ternary complex with hPNMT and the 3-hydroxymethyl-7-nitro-THIQ inhibitor (PDB: 2G70)³⁰ reveals a near identical conformation for the two ligands (Supporting Information Figure S3). As such, a methyl group was added to the SAH coordinates, and the structure was truncated as described above. The norepinephrine nucleophile was simplified to 1-amino-2-propanol, which was modeled in the neutral form. Previous studies indicate that even though norepinephrine may bind to

hPNMT as the ammonium species, deprotonation to the neutral amine occurs as a discrete step preceding the rate-limiting methyl transfer.¹⁰ Last, acetone was used as an implicit solvent model (PCM) in all TS structure optimizations to more closely mimic the dielectric environment of the enzyme active site.

Initially, dihedral angles of the ethyl groups in the SAM model were fixed to their crystallographic values to mimic geometric constraints imposed by the enzyme. Optimized TS structures were generated by holding the C_{Me}-N distance constant at 2.0 Å while varying the C_{Me}-S distance in 0.2 Å steps from 1.8 to 3.2 Å. TS structures at each fixed C_{Me}-S bond length were then reoptimized with the C_{Me}-N bond varied from 1.6 to 3.2 Å in 0.2 Å increments. TS structures providing calculated KIEs consistent with the intrinsic values were further refined by varying the C_{Me}-S and C_{Me}-N bond lengths along the axis of methyl transfer in 0.01 Å steps. In the final model, all dihedral angle constraints were released and the TS structure was reoptimized holding only the C_{Me}-S and C_{Me}-N bond lengths constant. The best match to intrinsic KIEs for hPNMT was obtained with C_{Me}-S and C_{Me}-N distances of 2.26 and 2.18 Å, respectively (Figure 2). Overall, the conformation of SAM in this TS model is similar to that of the substrate in cocrystal structures with hPNMT (Supporting Information Figure S4). The TS structure retains a single imaginary frequency (476i cm⁻¹) corresponding to motion of the methyl group along the S-C_{Me}-N axis at an angle of 179.4°. In addition, the total S-C_{Me}-N distance at the TS is 4.44 Å and summation of the C_{Me}-S and C_{Me}-N bond orders (0.62 and 0.31, respectively) provides a value <1.00 (Supporting Information Table S1), indicating a loose S_N2 TS structure with early character.

Himo and co-workers used DFT methods to build several TS structures for hPNMT, where the most developed model ("Model C(H⁺)") included the catalytically relevant active site residues Glu185 and Glu219, as well as bound water molecules.⁹ This complex TS model gave a good fit to the experimental rate constant for hPNMT and predicted a total S-C_{Me}-N distance of 4.42 Å (C_{Me}-S = 2.22 Å, C_{Me}-N = 2.20 Å), which is consistent with the reactant geometry of our simplified TS structure (Figure 2). Interestingly, the authors found that a truncated model of the hPNMT TS ("Model A"), similar to the structure presented in Figure 2, also provided a reasonable fit to the experimental rate constant using bond distances comparable to those observed in their complex model system.⁹ In a separate investigation, Liu *et al.* modeled the hPNMT reaction using QM/MM calculations that included the full protein solvated in a 30 Å water sphere.¹⁰ This more computationally intensive study considered three discrete TS structures for the hPNMT reaction: (1) a nonrate-limiting deprotonation of norepinephrine, (2) a rate-limiting methyl transfer step, and (3) a nonrate-limiting deprotonation of epinephrine. The authors report a more expanded TS structure for the methyl transfer step (S-C_{Me}-N distance = 4.66 Å);¹⁰ however, the early character (C_{Me}-S = 2.19 Å, C_{Me}-N = 2.47 Å) of this TS structure is consistent with the models reported by Himo and co-workers,⁹ as well as our simplified TS model (Figure 2). Comparison of the hPNMT TS structure to those of NSD2¹⁴ and DNMT1¹⁵ reveals similar distances along the methyl donor-acceptor axis (Supporting Information Table S1). Interestingly, hPNMT exhibits notably higher C_{Me}-S bond order (0.62) at the TS relative to NSD2¹⁴ and DNMT1¹⁵ (0.38 and 0.52, respectively), which supports an earlier TS for hPNMT and distinguishes this model from our previous TS structures.

Investigations on the catalytic mechanisms of COMT^{23,25} and GNMT²¹ have correlated the inverse α -secondary KIEs (i.e., $C_{Me}-^XH$) measured for these enzymes with increased out-of-plane bending vibrations imparted by compaction of the methyl donor–acceptor distance. By contrast, evidence from QM/MM^{31,32} and DFT³³ studies suggests that compression of the donor–acceptor distance in methyl transfer reactions may not be required to manifest such inverse α -secondary KIEs. In the TS search for hPNMT, all models giving close matches to the intrinsic KIEs had loose S_N2 character (i.e., summation of $C_{Me}-S$ and $C_{Me}-N$ bond orders was <1.00), and TS structures with compressed $S-C_{Me}-N$ distances gave poorer matches to the intrinsic primary *methyl*- ^{14}C and α -secondary 3H KIEs. Although the final TS for hPNMT matches the measured intrinsic KIEs (Table 1), it is important to note this model uses simplified reactant structures and does not include noncovalent contributions from enzyme active site. However, the loose character of the hPNMT TS is consistent with previous studies on the hPNMT reaction mechanism,^{9,10} as well as the TS structures of NSD2¹⁴ and DNMT1,¹⁵ all of which utilized more complex computational model systems.

Electrostatic potential maps extrapolated from single-point energy calculations (Gaussian 09, RB3LYP/6-31g(d) theory)²⁶ illustrate the distribution of partial positive (blue) and partial negative (red) charge for the hPNMT TS (Figure 3a) and the GS structure of SAM (Figure 3b). Whereas significant positive charge is localized on the sulfonium center of SAM in the GS, this positive charge is more evenly distributed between the sulfur of SAM, the transferring methyl group, and the incipient ammonium group of norepinephrine at the TS (Figure 3a,b). Natural bond orbital (NBO) calculations indicate that positive charge on the sulfur of SAM decreases by 0.35 at the TS, while the net positive charge on the transferring methyl group increases by 0.19 (Supporting Information Table S2). Overall, the TS model for hPNMT is consistent with those of NSD2¹⁴ and DNMT1,¹⁵ which retain a similar distribution of charge along the axis of methyl transfer. However, NBO values indicate that positive charge localization on sulfur of SAM is greater in the hPNMT TS (0.55) than in the models for NSD2¹⁴ and DNMT1¹⁵ (0.40 and 0.49, respectively). These data are consistent with the early character of the hPNMT TS shown by a direct comparison of bond lengths and bond orders for these models of SAM methyltransferases (Supporting Information Table S1).

Sinefungin (Supporting Information Figure S5) is a SAM analog and a pan-methyltransferase inhibitor that displays weak inhibition of rabbit PNMT.³⁴ Recently, a series of sinefungin-based inhibitors were reported to mimic TS features of the lysine methyltransferase SETD2.³⁵ The electrostatic potential map for sinefungin (Figure 3c) indicates significant positive charge is localized at the position of the transferring methyl group, a feature that does not accurately recapitulate the electrostatics of the PNMT TS structure. These data suggest sinefungin may not act to mimic the PNMT TS but rather function as a substrate analog. These models are consistent with the low binding affinity observed for sinefungin (lower than SAH product inhibition) with PNMT.³⁴

In conclusion, we have investigated the TS structure of hPNMT using a combination of experimental KIEs and DFT calculations. Intrinsic KIEs were determined for hPNMT at five atomic positions proximal to the transferring methyl group and employed as boundary constraints on KIE values predicted for calculated TS structures. The TS structure that best

matched the intrinsic KIEs was located at C_{Me}-S and C_{Me}-N distances of 2.26 and 2.18 Å, respectively. Our model indicates methyl transfer occurs *via* a rate-limiting S_N2 TS with early character, which is consistent with previous investigations on the catalytic mechanism of hPNMT. Electrostatic potential maps of the hPNMT TS structure illustrate an even distribution of positive charge along the axis of methyl transfer, a feature not reproduced by proposed methyltransferase TS analogs. Future work is focused on the design of chemically stable analogs that more accurately mimic the geometry and electronic configuration of the hPNMT TS structure.

METHODS

Full details for all experimental methods are provided in the Supporting Information.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

We thank Z. Wang (Einstein) for helpful discussions. This work was supported by research grant GM41916 from the National Institutes of Health.

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Figure 1.

The PNMT reaction and isotopically labeled SAM substrates used in the KIE measurements.

(a) PNMT catalyzes the SAM-dependent conversion of norepinephrine to epinephrine in the catecholamine biosynthetic pathway. (b) KIE measurements were carried out using a family of isotopically labeled SAM substrates. The colored labels illustrate where individual isotopes were incorporated into separate SAM molecules. PNMT, phenylethanolamine *N*-methyltransferase; SAM, *S*-adenosyl-L-methionine; SAH, *S*-adenosyl-L-homocysteine; KIE, kinetic isotope effect.

Figure 2.

Calculated model of the hPNMT TS structure. A model of the hPNMT TS structure was generated by matching calculated KIEs (Gaussian 09, RB3LYP/6-31g(d) theory)²⁶ to measured intrinsic values (Table 1). The best match between calculated and intrinsic KIEs was identified with C_{Me}-S and C_{Me}-N distances of 2.26 and 2.18 Å, respectively. hPNMT, human phenylethanolamine *N*-methyl-transferase; TS, transition state; KIEs, kinetic isotope effects; Me, methyl. Atom colors are hydrogen, white; carbon, black; oxygen, red; nitrogen, blue; sulfur, yellow.

Figure 3.

Electrostatic potential map for the hPNMT TS model. Electrostatic potential maps (red = partial negative; blue = partial positive) for optimized structures (Gaussian 09, RB3LYP/6-31g(d) theory)²⁶ were extrapolated from single-point energy calculations and visualized in GaussView 5.0 (isovalue = 0.04). (a) Electrostatic potential map for the hPNMT transition state model presented in Figure 2. (b) Electrostatic potential map for the C_{Me}-S bond of SAM in the GS. (c) Electrostatic potential map for the R₂HC-NH₃ bond of sinefungin. hPNMT, human phenylethanolamine *N*-methyltransferase; TS, transition state; GS, ground state; SAM, *S*-adenosyl-L-methionine.

Table 1

V/K and Intrinsic KIEs for the hPNMT Reaction

heavy SAM	light SAM	type of KIE	V/K KIEs	intrinsic KIEs ^c	calculated KIEs ^d
[Me- ¹⁴ C]	[1'- ³ H]	primary	1.108 ± 0.004 ^b	1.116 ± 0.005	1.119
[³⁶ S, 8- ¹⁴ C]	[1'- ³ H]	primary	1.013 ± 0.004 ^b	1.014 ± 0.005	1.015
[Me- ³ H ₂]	[8- ¹⁴ C] ^a	α -secondary	0.810 ± 0.005	0.796 ± 0.006	0.797
[5'- ¹⁴ C]	[1'- ³ H]	α -secondary	0.996 ± 0.008 ^b	0.996 ± 0.009	1.001
[5'- ³ H ₂]	[8- ¹⁴ C]	β -secondary	1.043 ± 0.005	1.047 ± 0.006	1.043
[1'- ³ H]	[8- ¹⁴ C]	remote	1.013 ± 0.001	1.013 ± 0.002	

^aThe KIE on the remote [8-¹⁴C] label is assumed to be unity.^bExperimental values were corrected for the remote [1'-³H] KIE using the expression $KIE = KIE_{obs} \times [1'-^3H]KIE$.^cIntrinsic KIEs were determined by correcting V/K values using eq 1 assuming $C_T^1 = 0.074 \pm 0.001$.^dCalculated values (Gaussian 09, RB3LYP/6-31g(d) theory)²⁶ correspond to those for the TS model shown in Figure 2.hPNMT, human phenylethanolamine *N*-methyltransferase; SAM, *S*-adenosyl-L-methionine; KIEs, kinetic isotope effects; Me, methyl.