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Transition-State Structure of Human 5′-Methylthioadenosine Phosphorylase

Vipender Singh and Vern L. Schramm*

Contribution from the Department of Biochemistry, Albert Einstein College of Medicine, Bronx, New York 10461

Received July 27, 2006; E-mail: vern@aecom.yu.edu

Abstract: Kinetic isotope effects (KIEs) and computer modeling using density functional theory were used to approximate the transition state of human 5′-methylthioadenosine phosphorylase (MTAP). KIEs were measured on the arsenolysis of 5′-methylthioadenosine (MTA) catalyzed by MTAP and were corrected for the forward commitment to catalysis. Intrinsic KIEs were obtained for [1′-^{3}H], [1′-^{14}C], [2′-^{3}H], [4′-^{3}H], [5′-$^{3}H_2$], [9-^{15}N], and [Me-$^{3}H_3$] MTAs. The primary intrinsic KIEs (1′-^{14}C and 9-^{15}N) suggest that MTAP has a dissociative S_N1 transition state with its cationic center at the anomeric carbon and insignificant bond order to the leaving group. The 9-^{15}N intrinsic KIE of 1.039 also establishes an anionic character for the adenine leaving group, whereas the α-primary 1′-^{14}C KIE of 1.031 indicates significant nucleophilic participation at the transition state. Computational matching of the calculated EIEs to the intrinsic isotope effects places the oxygen nucleophile 2.0 Å from the anomeric carbon. The 4′-^{3}H KIE is sensitive to the polarization of the 3′-OH group. Calculations suggest that a 4′-^{3}H KIE of 1.047 is consistent with ionization of the 3′-OH group, indicating formation of a zwitterion at the transition state. The transition state has cationic character at the anomeric carbon and is anionic at the 3′-OH oxygen, with an anionic leaving group. The isotope effects predicted a 3′-endo conformation for the ribosyl zwitterion, corresponding to a H1′−C1′−C2′−H2′ torsional angle of 33°. The [Me-$^{3}H_3$] and [5′-$^{3}H_2$] KIEs arise predominantly from the negative hyperconjugation of the lone pairs of sulfur with the σ^* (C−H) antibonding orbitals. Human MTAP is characterized by a late S_N1 transition state with significant participation of the phosphate nucleophile.

Introduction

Kinetic isotope effects (KIEs) are the method of choice for studying the transition states of enzymatic reactions and have been used to establish the properties of transition states of *N*-ribosyltransferases for purine and pyrimidine nucleosides.[1−3] In the KIE approach used here, heavy isotopes (^{3}H, ^{14}C, and ^{15}N) are substituted at positions expected to show bond vibrational differences on conversion of reactants to the transition state. To determine the transition-state structure of an enzymatic reaction, (k_{cat}/K) KIEs are corrected for the commitment factors to obtain intrinsic KIEs, that is, KIEs on the bond-breaking step. The intrinsic KIEs originate from the vibrational difference between the free substrate in solution and at the transition state. KIEs provide a boundary condition for computational modeling of the enzymatic transition state. The transition state for an enzyme-catalyzed reaction is approximated by correlating the calculated KIEs with the intrinsic KIEs.

Most *N*-ribosyltransferases have dissociative S_N1 transition states which are characterized by the formation of a ribosyl oxacarbenium ion with increased positive charge on the anomeric carbon and decreased negative charge on the ribosyl ring oxygen. Among the few exceptions is the transition state of thymidine phosphorylase, which has an S_N2 mechanism.[2] Another common feature of the *N*-ribosyltransferases is that dissociation of the *N*-glycosidic bond is accompanied by an increase in the pK_a of the leaving group. An active-site general acid (Asp197 for *Escherichia coli* 5′-methylthioadenosine nucleosidase (MTAN[4]) and Asp198 in *Mycobacterium tuberculosis* purine nucleoside phosphorylase (PNP[6])) is often present to protonate N7 of the leaving group and stabilize the transition state. Transition-state analyses of MTANs have shown that N7 is protonated at the transition state of *E. coli* MTAN but not at the transition state of *Streptococcus pneumoniae* MTAN. The higher activation barrier for the *S. pneumoniae* MTAN is reflected in a k_{cat} for *S. pneumoniae* MTAN of 0.25 s^{-1}, 16-fold less than that of *E. coli* MTAN.[4,5]

In this study, the transition state of human 5′-methylthioadenosine phosphorylase (MTAP) is explored by KIE measurements and computational modeling using density functional methods implemented in Gaussian 03.[7] MTAP is a purine salvage enzyme found in mammals. It catalyzes the reversible phosphorolysis of the *N*-glycosidic bond of MTA to form 5′-

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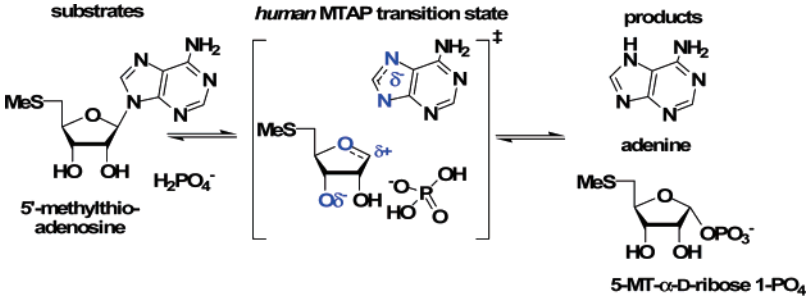
Scheme 1. Phosphorolysis of MTA by Human MTAP and the Proposed Transition State[a]



[a] Atoms with differences in charge at the transition state are shown in blue. Details of this transition state are presented in Table 2. KIE experiments used arsenate to reduce commitment factors.

methylthioribose1-phosphate (MTR-1-P) and adenine (Scheme 1). Disruption of MTAP has been shown to affect purine salvage and methionine and polyamine pathways, and it has been proposed to be an anti-cancer drug target.[8−10] This study suggests that human MTAP has a dissociative S_N1 transition state, and in this respect it is similar to other members of the *N*-ribosyltransferse family. However, the ribosyl group at the transition state of MTAP exists as a stabilized zwitterion, cationic at the anomeric carbon and anionic at the oxygen of the 3′-hydroxyl. The positive charge on the anomeric carbon at the transition state is stabilized by low bond order to the attacking anionic phosphate or arsenate nucleophile. Further, the N7 of adenine is not protonated at the transition state. The catalytic acceleration therefore comes from the stabilization of the methylthioribosyl zwitterion by a phosphate anion and distortion or ionization of the 3′-OH group.

Material and Methods

Expression and Purification of Human MTAP. Details of the DNA manipulation, protein expression, and purification procedure for human MTAP have been described previously.[11] Briefly, the enzyme was overexpressed in *E. coli* using the pQE32 expression vector. The overexpressed MTAP, His_6-tagged at the N-terminus, was purified using a Ni−NTA resin column with a 30−300 mM imadizole gradient. The purified protein was concentrated, dialyzed against 100 mM Tris, pH 7.9, 50 mM NaCl, and 2 mM dithiothreitol (DTT), and stored at −80 °C.

Enzymes and Reagents for MTA Synthesis. The reagents and the enzymes used in the synthesis of MTAs from glucose have been described previously elsewhere.[1]

Synthesis of Radiolabeled MTAs. Isotopically labeled [1′-^{3}H]MTA, [1′-^{14}C]MTA, [2′-^{3}H]MTA, [3′-^{3}H]MTA, [4′-^{3}H]MTA, [5′-$^3H_2$]MTA, [*methyl*-$^3H_3$]MTA, and [8-^{14}C]MTA were synthesized from the corresponding ATP molecules in two steps using the procedure described elsewhere.[1]

Measurement of Kinetic Isotope Effects. The KIEs were measured by mixing ^{3}H- and ^{14}C-labeled substrates with ^{3}H:^{14}C in a 4:1 ratio. The MTAP assays for measuring KIEs were performed in triplicates of 1 mL reactions (100 mM Tris-HCl, pH 7.5, 50 mM KCl, 250 μM MTA (including label), 15 mM sodium arsenate, and 1.0−5.0 nM human MTAP) containing $>10^5$ cpm of ^{14}C. After 20−30% completion of the reaction, 750 μL of the reaction was resolved on charcoal−Sepharose (acid-washed powdered charcoal and Sepharose in 1:4 ratio made into a slurry in 1 mM 5-methylthioribose (MTR) and settled in Pasteur pipets). The remainder of the reaction mixture was allowed to react to completion and then applied to the column. Columns were washed with two volumes of 1 mM MTR, and radioactive methylthioribose was eluted with six volumes of 15 mM MTR containing 50% ethanol. Each 1.0 mL of eluate was mixed with 9.0 mL of scintillation fluid and counted for at least three cycles at 10 min per cycle. The ^{3}H:^{14}C ratio was determined for partial and complete reactions, and the KIEs were corrected to 0% hydrolysis by the equation

$$\mathrm{KIE} = \frac{\ln(1-f)}{\ln\left[\left(1-f\frac{R_f}{R_o}\right)\right]}$$

where f is the fraction of reaction progress and R_f and R_o are the ratios of heavy to light isotope at partial and total completion of reaction, respectively.

Forward Commitment Factor. The isotope partition method[12] was used to measure the forward commitment to catalysis. The 20 μL "pulse solution", containing 20 μM human MTAP in 100 mM Tris, pH 7.5, 50 mM KCl, 1 mM DTT, and 200 μM [8-^{14}C]MTA containing 10^5 cpm, was incubated for 10 s and then diluted with 180 μL of "chase solution", containing a large excess of unlabeled MTA (2.6 mM) in 100 mM Tris, pH 7.5, 50 mM KCl, 1 mM DTT, and various concentrations of sodium arsenate (0.1, 0.3, 0.5, 1.0, 2.0, 4.0, 7.0, 20, and 40 mM). The samples were incubated for 20 s to allow a few turnovers and then quenched with 1 N HCl. Product formation was measured by reverse-phase HPLC using a C-18 Deltapak column by 25% methanol and in 50 mM ammonium acetate, pH 5.0, and scintillation counting. The forward commitment to catalysis is calculated from the fraction of bound MTA converted to product following dilution in excess MTA. This procedure uses [8-^{14}C]MTA as a stoichiometric label for the catalytic site, and the commitment factors are independent of any KIE, although none would be expected from [8-^{14}C]MTA.

Computational Modeling of the Transition State. The in vacuo determination of the MTAP transition state used hybrid density functional methods implemented in Gaussian 03.[7] The substrate and the transition state were modeled using the one-parameter Becke (B1) exchange functional, the LYP correlation functional, and the 6-31G-(d,p) basis set.[6] The same level of theory and basis set were also used for the computation of bond frequencies. During the calculations, the 5′-methylthio group was constrained by freezing the O4′−C4′−C5′−S and C4′−C5′−S−C^{Me} torsion angles. The properties of the leaving group at the transition state were modeled separately.

Equilibrium isotope effects (EIEs) were calculated from the computed frequencies of the substrate and the transition-state using ISOEFF 98 software.[13a] All $3N-6$ vibrational modes were used to calculate the

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isotope effects, but only those that exhibit shifts due to the isotopic substitution contribute to isotope effects. The isotope effects were calculated at 298 K.

The applied geometric constraints were optimized iteratively to generate a transition-state model for which the primary and the β-secondary EIEs closely match the intrinsic KIEs. The seconday intrinsic KIEs at other positions were then explored systematically to obtain group properties that matched the experimental intrinsic KIEs. Constrained molecules impose energetically unfavorable positions relative to vacuum conditions for transition-state searches. These reflect the forces imposed by the enzymatic environment. Clearly, this approach yields an approximation of the transition state. Frequencies for unconstrained and constrained transition states are provided in the Supporting Information.

The contribution of solvent to the state of the free reactant has been tested in a closely related reaction, hydrolysis of the *N*-ribosidic bond of 5′-methylthioadenosine by *S. pneumoniae* methylthioadenosine nucleosidase. The effects of changing the implicit solvent (by changing the empirical parameter of the dielectric constant) on isotope effects were examined by the self-consistent reaction field (SCRF) method using the polarization continuum model for 5′-methylthioadenosine at the transition state. Changing the dielectric constant from 4.9 (for chloroform) to 78.8 (for water) has no effect on the calculated EIEs (Singh, V., and Schramm, V. L., submitted for publication). Explicit solvent water interactions are also unlikely to influence KIEs by hydroxyl group interactions. Gawlita et al. have shown that desolvation of primary and secondary hydroxyls does not cause isotope effects on the neighboring CH bonds.[13b] On the basis of these analyses, no corrections for solvent interactions have been applied.

The natural bond orbital (NBO) calculations were performed on optimized structures by including the pop = (nbo, full) keyword in the route section of input files, and the molecular electrostatic potential (MEP) surfaces were visualized using Molekel 4.0.[14]

Results and Discussion

Experimental Kinetic Isotope Effects (KIEs) and Commitment Factors. Human MTAP catalyzes the reversible phosphorolysis of the *N*-glycosidic bond of MTA to 5-methylthioribose 1-phosphate and adenine. To avoid kinetic complexity associated with the transition-state analyses for reversible reactions, the KIEs for the human MTAP were measured on the physiologically irreversible reaction of arsenolysis. The products of arsenolysis are adenine and 5-methylthioribose 1-arsenate. Methylthioribose 1-arsenate is unstable and rapidly decomposes to form methylthioribose and arsenate. Although the possibility of an on-enzyme equilibrium cannot be ruled out, an intrinsic KIE of 1.039 for ^{15}N9 (within experimental error of the theoretical maximum of 1.036 for complete dissociation of the *N*-glycosidic bond[15a]) and a large 1′-^{3}H (the largest reported for any *N*-ribosyltransferases) preclude the existence of such an equilibrium. Therefore, the intrinsic KIEs reported in Table 1 are not reduced by reverse commitment. The KIEs were measured for MTAs labeled at [1′-^{3}H], [1′-^{14}C], [2′-^{3}H], [4′-^{3}H], [5′-^{3}H$_2$], [9-^{15}N], and [Me-^{3}H$_3$] using competitive conditions. [5′-^{14}C]MTA was used as a remote control for measuring tritium isotope effects, and [5′-^{3}H$_2$]MTA was used as the remote label to measure 1′-^{14}C and 9-^{15}N/5′-^{14}C KIEs. The 1′-^{14}C and 9-^{15}N/5′-^{14}C KIEs were corrected for the 5′-^{3}H$_2$ KIE. The measured KIEs were also corrected for the external forward commitment of 0.265 ± 0.027 (Figure 1) using Northorp's equation modified for irreversible reactions: $^T(V/K) = (^Tk + C_f)/(1 + C_f)$, where $^T(V/K)$ is an observed tritium isotope effect and C_f is the forward commitment to catalysis. The intrinsic KIEs were obtained by correcting the observed KIEs (column 3, Table 1) for the forward commitment.

Table 1. KIEs Measured at pH 7.5 for Arsenolysis of MTA by Human MTAP

		KIEs		
substrate	type of KIE	exptl[a]	intrinsic[d]	calcd
[1′-^{3}H]	α-secondary	1.290 ± 0.003	1.360 ± 0.003	1.200
[1′-^{14}C]	primary	1.025 ± 0.005[b]	1.031 ± 0.005	1.030
[2′-^{3}H]	β-secondary	1.063 ± 0.003	1.079 ± 0.003	1.076
[9-^{15}N/5′-^{14}C]	primary	1.031 ± 0.002[c]	1.039 ± 0.002	1.036
[4′-^{3}H]	γ-secondary	1.037 ± 0.005	1.047 ± 0.005	0.997
[5′-^{3}H]	δ-secondary	1.038 ± 0.001	1.048 ± 0.001	1.050
[Me-^{3}H]	remote	1.073 ± 0.002	1.092 ± 0.002	1.028

[a] Experimental KIEs are corrected to 0% substrate depletion. [b] The 1′-^{14}C KIE was corrected for the 5′-^{3}H KIE according to expression KIE = KIE$_{observed}$ × 5′-^{3}H KIE. [c] The 9-^{15}N KIE was corrected for 5′-^{3}H KIE according to expression KIE = KIE$_{observed}$ × 5′-^{3}H KIE. [d] Intrinsic KIE values are corrected for the forward commitment shown in Figure 1.

Computation of the Transition State. The transition state of human MTAP was modeled using the B1LYP functional and 6-31G(d,p) basis sets. The modeling was performed using a 5-methylthioribosyl oxacarbenium ion, anionic adenine as a leaving group, and a neutral phosphate nucleophile. The calculated KIE values were tried with both arsenate and phosphate, and the differences were within the standard error limits of the experimental KIEs; therefore, we elected to use phosphate, the physiological nucleophile, in the computational analysis. The initial transition-state model generated by an in vacuo calculation without imposing any external constraints predicted an S_N2-like transition state, which is characterized by a large 1′-^{14}C KIE with significant bond order to the leaving group and the phosphate nucleophile. It had a single imaginary frequency of 295i cm^{-1} (see Supporting Information). The experimental intrinsic KIE of 1.031 for [1′-^{14}C]MTA suggests that the anomeric carbon has a small but significant bond order to either the leaving group or an attacking phosphate nucleophile, or to both, at the transition state. The 1′-^{14}C KIE, together with the 9-^{15}N intrinsic KIE of 1.039, which is consistent with the complete dissociation of the *N*-glycosidic bond at the transition state, suggest that the 1′-^{14}C KIE of 1.031 arises entirely from increased bonding to the phosphate oxygen nucleophile. Therefore, in the subsequent modeling of the MTAP transition state, additional distance constraints were applied to the leaving group and to the phosphate nucleophile. The 5-methylthio group, being away from the reaction center, was constrained by fixing the O4′−C4′−C5′−S and C4′−C5′−S−C^{Me} torsional angles. The calculations were performed by increasing the C1′−N9 distance in 0.1 Å increments up to 4.0 Å, where the bond order is negligible. The leaving group was not included in subsequent calculations. Application of bond constraints to the transition state resulted in the appearance of two imaginary frequencies

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Table 2. Geometric and Electronic Changes in Representative Models of the Substrate and the Transition State Calculated Using B1LYP/6-31G** (human_MTAP)

	bond length (Å)		bond order charge[a]	hyperconjugation (kcal/mol)[b] substrate		hyperconjugation (kcal/mol)[b] TS			orbital changes[c]			
bond type	GS	TS	Δ(σ − σ*)	σ→	→σ*	σ→	→σ*	Δ(TS − GS)	GS hybrid	GS carbon content (%)	TS hybrid	TS carbon content(%)
C1′−H1′	1.0936	1.0848	−0.01584	10.95	8.10	7.12	6.97	−1.28	$sp^{2.81}$	63.04	$sp^{1.81}$	64.22
C2′−H2′	1.0973	1.0980	+0.01244	5.37	11.23	12.65[d]	7.93	−0.11	$sp^{2.72}$	62.09	$sp^{2.86}$	63.72
C4′−H4′	1.0964	1.0916	−0.00471	7.02	11.50[e]	8.44	5.59	3.60	$sp^{2.91}$	62.45	$sp^{2.83}$	63.98
C1′−N9	1.4582	na	na	8.84	28.64	na	na	na	$sp^{3.27}$	35.70	na	na
C5′−H5′ (*R*)	1.0910	1.0933	-0.00232	5.39	5.66	6.44	4.88[g]	4.39	$sp^{3.04}$	63.86	$sp^{3.10}$	64.04
C5′−H5′ (*S*)	1.0914	1.0912	−0.00337	5.61	4.21[h]	4.01	4.52	−0.30	$sp^{3.02}$	63.42	$sp^{2.80}$	64.35
C^S−H(A)	1.0920	1.0906	−0.00208	3.39	0.89	0.67	4.63[i]	1.18	$sp^{3.02}$	62.71	$sp^{2.77}$	62.59
C^S−H(B)	1.0893	1.0907	−0.00205	<0.50	4.31	2.87	1.04[i]	0.01	$sp^{2.78}$	62.71	$sp^{2.92}$	63.78
C^S−H(C)	1.0872	1.0906	−0.00360	0.50	5.27[j]	<0.50	3.85[i]	−1.81	$sp^{2.74}$	62.22	$sp^{2.76}$	63.25
C3′−H3′	1.0908	1.0941	−0.00103	6.62	9.42	5.60	8.02[k]	1.29	$sp^{2.67}$	63.49	$sp^{2.75}$	64.17

[a] Calculated by subtracting the number of electrons occupying the σ* orbital from the number occupying the σ orbital and listed as change between substrate and transition state (substrate − TS). [b] Sum of second-order perturbation contributions calculated by NBO analysis. Cutoff = 0.5 kcal/mol. [c] Hybridization of the carbon atom and contribution of the carbon atom to the bond in percent. GS is the ground state of the substrate, and TS is the transition state. [d−k] Lp1 is the sp-type lone pair, and Lp2 is p-type lone pair: [d]Lp2(C1′), [f]Lp1(O3′), [g]Lp2(S), [i]Lp2(S), and [k]Lp2(O3′) are better acceptors in the transition state, while [e]Lp2(O4′), [h]Lp2(S), and [j]Lp2(S) are better acceptors in the substrate.

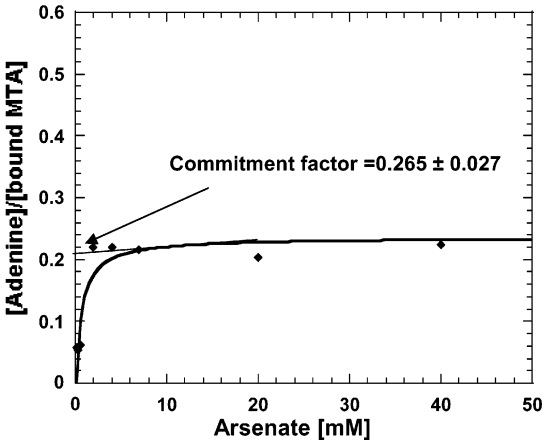

Figure 1. Forward commitment to catalysis for the MTAP−MTA complex. The complex of human MTAP and [^{14}C]MTA was diluted with a large excess of unlabeled MTA and varying concentrations of sodium arsenate. The subsequent reaction partitions bound [^{14}C]MTA to product (forward commitment) or permits release into free, unbound MTA. Zero commitment extrapolates through the origin. The forward commitment was calculated by plotting the amount of labeled adenine formed following addition of chase solution, containing saturating amounts of sodium arsenate, divided by the amount of labeled MTA on the active site before dilution with chase solution. The line is drawn from an ordinary least-squares fit of the data to the Michaelis−Menten equation. The intercept value is 0.21 and the forward commitment factor were calculated from the intercept using the expression (intercept/1 − intercept). The forward commitment to catalysis for human MTAP is 0.265 ± 0.027.

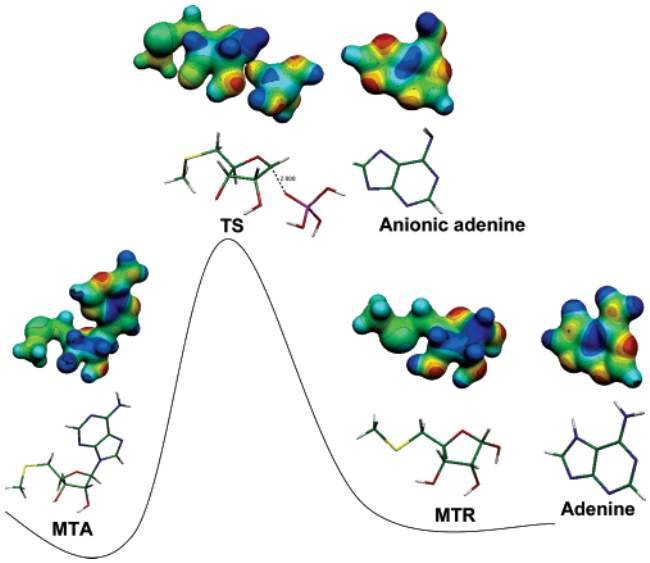

Figure 2. Reaction coordinate cartoon for the arsenolysis of MTA by MTAP. Molecular electrostatic potential surfaces (MEPs) are shown for the MTA reactant, transition state, and products. The transition state is shown as a fully dissociated anionic adenine, and a ribosyl−phosphate interaction is shown at 2.0 Å, assuming that arsenate and phosphate are equivalent in transition-state interactions. MEPs were calculated at the HF/STO3G level (Gaussian 98/cube) for the geometry optimized at the B1LYP/6-31G(d,p) level of theory and visualized with Molekel 4.0 at a density of 0.05 electron/Å^3. The stick models have the same geometry as the MEP surfaces. The ribosyl 3-hydroxyl is ionized to the anion. The ribosyl-arsenate hydrolyzes following bond formation, and the products are shown as the hydrolysis products of MTR and neutral adenine.

(see Supporting Information). The in vacuo transition state of an unconstrained reaction is the highest point on the potential energy surface (PES) and is characterized by a single imaginary frequency. The enzymatic PES is expected to differ from that in vacuo. The enzymatic transition-state model is generated by correlating the theoretical KIEs to intrinsic KIEs. This coincidence locates the transition state for the enzymatic PES. This structure is no longer a transition state or a maximum on the in vacuo PES and hence has more than one imaginary frequency. Frequencies are obtained from the second-derivative matrix of potential energy with respect to Cartesian coordinates. For the dissociative S_N1 transition state with no significant bond order to the *N*-glycosidic bond, the relatively small imaginary frequencies have little effect on the primary (1′-^{14}C and 9-^{15}N) KIEs. Therefore, the calculated KIEs were similar to the EIEs. Since the intrinsic KIEs were closely related to a fully dissociated, stabilized ribooxacarbenium ion, the subsequent TS models were optimized as a TS intermediate. All $3N - 6$ normal modes of the transition-state model and the substrate were used to calculate EIEs using ISOEFF 98.[13] The applied external constraints were iteratively optimized until the calculated EIEs were correlated with the intrinsic KIEs. In the transition state, the oxygen of the phosphate nucleophile is 2.0 Å from the anomeric carbon. The leaving group was modeled separately and is discussed below with 9-^{15}N KIE, along with the properties of the transition state.

Intrinsic 9-^{15}N, 1′-^{14}C, and 1′-^{3}H KIEs. The 9-^{15}N intrinsic KIE of 1.039 measured for human MTAP is close to the theoretical maximum ^{15}N isotope effect of 1.040 and also within

experimental error of the 9-^{15}N isotope effect of 1.036 calculated for the complete dissociation of the *N*-glycosidic bond.[15] Calculations on the adenine leaving group to study the effect of protonation at nitrogens N1, N3, N7, and N9 on the 9-^{15}N isotope effect show that the protonation of N7 decreases the 9-^{15}N isotope effect from 1.036 to 1.025.[15] Therefore, an intrinsic KIE of 1.039 for [9-^{15}N]MTA suggests that the dissociation of the *N*-glycosidic bond is complete and the N7 is not protonated at the transition state of human MTAP. The activation of the leaving group in the form of N7 protonation is a recurrent feature in the transition states of *N*-ribosyltransferases. Among the few exceptions are the transition states of *S. pneumoniae* MTAN and a mutant adenosine 5′-monophosphate nucleosidase.[16] Therefore, protonation of N7 is not required for cleavage of the *N*-glycosidic bond, and the catalytic acceleration originates from formation of a methylthioribose cation at the transition state.

Crystallographic evidence also suggests that leaving group activation in the form of protonation of N7 is modest in MTAP. Crystal structures of human MTAP with MTA (its substrate, not protonated at N7) and MT-ImmA (a transition-state analogue with protonated N7) show equivalent O^{Asp220}−N7 distances within the crystallographic errors in these two structures (3.0 Å in the MTA structure and 2.9 Å in the structure of MT-ImmA with human MTAP).[9] However, the ionization of Asp220 is not revealed by crystallography and could make an important difference in the binding energies of MTA and MT-ImmA.

The primary 1′-^{14}C intrinsic KIE is the most useful probe for determining the mechanism of nucleophilic substitution reactions (S_N1 vs S_N2) of *N*-ribosyltransferases.[17] A 1′-^{14}C KIE of 1.00−1.03 indicates dissociative S_N1 transition states, 1.03−1.08 indicates significant associative interactions in S_N1 transition states, and >1.08 indicates the properties of an S_N2 transition state with a neutral reaction center (anomeric carbon in the case of ribosyltransferases). An intrinsic KIE of 1.031 for human MTAP indicates an S_N1 transition state with significant bond order to the phosphate nucleophile. The transition state consistent with the kinetic isotope effects predicted a C1′−$O^{phosphate}$ bond distance of 2.0 Å. The small primary 1′-^{14}C KIE indicates a change in hybridization at the anomeric carbon, from $sp^{2.83}$ in the substrate to $sp^{2.40}$ at the transition state. These changes cause increased cationic character at the transition state (positive charges on O4′ and C1′ increase by +0.20 and +0.25, respectively) relative to the reactant state. This sharing of charge is characteristic of ribooxacarbenium ions.[17] The change in hybridization also creates a partially empty $2p_z$ orbital on C1′ that hyperconjugates with the σ(C2′−H2′) electrons and lone pair of O4′ and stabilizes the transition state by partially neutralizing the positive charge on C1′.

The large 1′-^{3}H intrinsic KIE of 1.360 is consistent with the dissociative S_N1 transition state, as indicated by the 1′-^{14}C and 9-^{15}N KIEs. The large 1′-^{3}H KIE arises mainly from a substantial decrease in bending frequencies for the out-of-plane bending modes due to increase steric freedom of C1′−H1′ following dissociation of the C1′−N9 bond. The 1′-^{3}H KIE is also influenced by van der Waals interactions with active-site residues and by the orientation of the base in the reactant MTA.[15a] Polarization of the 2′-hydroxyl and rotation of the H1′−C1′−C2′−H2′ and H2′−C2′−O−H torsion angles also have a small influence on the 1′-^{3}H KIE.[14] Although all these factors are difficult to model together, the large 1′-^{3}H KIE is consistent with the dissociative transition state. Quantum mechanical tunneling is known to influence ^{3}H secondary kinetic isotope effects in hydride-transfer reactions[15b] but is unlikely to be coupled to the reaction coordinate motion of C−N bond cleavage. We have therefore ignored possible contributions from H-tunneling.

The 4′-^{3}H KIE and Evidence for a 5′-Methylthioribosyl Zwitterion. For dissociative S_N1 transition states, theoretical calculations predict a large inverse isotope for [4′-^{3}H]MTA.[15a] This arises from the interaction of the σ(C4′−H4′) bond with the relatively electron-deficient O4′ and cationic center at the anomeric carbon.[1,15a] Previously, normal intrinsic 4′-^{3}H KIEs of 1.012 and 1.010 were measured for *S. pneumoniae* and *E. coli* MTANs.[1,15a] In those cases, the polarization of the 3′-OH increases the electron density on the ring oxygen (O4′) due to unequal charge sharing, causing the hyperconjugation of the lone pair of O4′ to the σ^*(C4′−H4′) antibonding orbital to increase to give a normal 4′-^{3}H KIE isotope effect. Glu174 was recognized as the residue responsible for the polarization of 3′-OH in MTANs.[1,15a] Human MTAP also has a larger than normal intrinsic 4′-^{3}H KIE of 1.047, suggesting a similar mechanism. The crystal structure of human MTAP with MT-ImmA (a transition-state analogue) shows that one of the phosphate oxygens is strongly hydrogen bonded to the 3′-OH ($O^{hydroxyl}$−$O^{phosphate}$ distance 2.6 Å). Ionization of the 3′-hydroxyl creates an anionic center at this oxygen. The transition state therefore is zwitterionic, with a partial positive charge on the anomeric carbon and a negative charge on the oxygen of the 3′-hydroxyl.[15c]

The 4′-^{3}H intrinsic KIE of 1.047 for human MTAP is also influenced by the phosphate nucleophile at the transition state. Participation of phosphate partially neutralizes the positive charge on the anomeric carbon and increases the occupancy of the partially empty p orbital on the anomeric carbon due to increased bonding character between the anomeric carbon and the oxygen of a phosphate nucleophile. The occupancy of the $2p_z$ orbital increases from 0.65 for *S. pneumoniae* MTAN to 0.85 in human MTAP, whereas the positive charge on the anomeric carbon decreased from 0.58 in *S. pneumoniae* MTAN to 0.55 in human MTAP. These changes increase the intrinsic 4′-^{3}H KIE to 1.047 in human MTAP, compared to values of 1.012 and 1.010 as measured for *S. pneumoniae* and *E. coli* MTAN, respectively.[1,15a] The increased occupancy of the p orbital as well as the partial neutralization of the positive charge on the anomeric carbon causes the n_p (O4′)-to-p orbital (C1′) hyperconjugation to decrease, and there is a corresponding increase in the n_p (O4′)-to-σ^*(C4′−H4′) bond.

The phosphate nucleophile is also hydrogen-bonded to both the 2′-hydroxyl and the 3′-hydroxyl of MTA/MT-ImmA in the active site of human MTAP.[9] In the crystal structure of MTA with sulfate (an analogue of phosphate), the O−O bond distance between the oxygens of sulfate and the 2′-hydroxyl and the 3′-hydroxyl are 3.0 and 2.4 Å, respectively. These distances change to 2.8 and 2.6 Å, respectively, in the crystal structure of human MTAP with MT-ImmA (a transition-state analogue). As the reaction approaches the transition state, the O−O bond distance between the oxygen of the 3′-hydroxyl and the nucleophile appears to increase, with a decrease in the O−O bond distance between the 2′-hydroxyl and the phosphate nucleophile. The

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KIE analysis also supports the movement of a nucleophile toward the anomeric carbon and away from the 3′-hydroxyl. Transition-state analysis suggests that ionization of the 3′-hydroxyl results from motion relative to the basic phosphate molecule. Formation of an anion at the 3′-hydroxyl stabilizes a water molecule observed in the crystal structure of the transition-state analogue with human MTAP. This interaction is absent in the crystal structure of human MTAP with the MTA substrate.[18]

Kinetic analysis with substrate analogues provides additional evidence concerning the importance of the 3′-hydroxyl in catalysis.[19] The k_{cat}/K_m of human MTAP for MTA is 3.2×10^6 M^{-1} s^{-1}. It decreases to 9×10^4 M^{-1} s^{-1} for 2′-deoxy-MTA and to 10^3 M^{-1} s^{-1} for 3′-deoxy-MTA. This change is predominantly a k_{cat} effect, which decreases from 4.6 s^{-1} for MTA to 0.28 and 0.004 s^{-1} for 2′- and 3′-deoxy-MTA, respectively, whereas the K_m only increased ∼2-fold. The ∼1000-fold decrease in k_{cat} for 3′-deoxy-MTA relative to MTA suggests that the oxygen of the 3′-hydroxyl is important in stabilizing the transition state. The formation of an oxyanion from ionization of the 3′-hydroxyl further stabilizes the positive charge at the anomeric carbon.

The 2′-^{3}H KIE and Ribosyl Puckering. The positive hyperconjugation of σ(C2′−H2′) bonding electrons to a partially empty $2p_z$ orbital on the anomeric carbon at the transition state is the predominant factor that influences the magnitude of the 2′-^{3}H KIE.[15a] The contribution of this hyperconjugation to the total 2′-^{3}H intrinsic KIE is dependent on the ribose puckering at the transition state. Its magnitude depends on the extent of overlap between the C2′−H2′ σ bond and the $2p_z$ orbital. The magnitude of this effect varies as a $\cos^2\theta$ function of this overlap.[15a] If the entire 2′-^{3}H KIE originates from the hyperconjugative interaction between σ(C2′−H2′) and the p ortibal, an intrinsic 2′-^{3}H KIE of 1.079 for human MTAP corresponds to the H2′−C2′−C1′−H1′ torsion angle of 33° and a small 3-endo pucker corresponding to the O4′−C1′−C2′−C3′ torsion angle of −13°. However, the 2′-^{3}H KIE is also influenced by polarization of the 2′-OH and 3′-OH and rotation of the H2′−C2′−O−H torsional bond, whereas only positive hyperconjugation is influenced by puckering of the ribose.[15a] To determine the fraction of the 2′-^{3}H intrinsic KIE that comes exclusively from σ(C2′−H2′)-to-$2p_z$ transfer, a calculation was performed by constraining the ribose sugar O4′−C1′−C2′−C3′ torsion angle to a value obtained from the crystal structure of MT-ImmA (a transition-state analogue inhibitor of human MTAP[9]) with human MTAP, leaving the H2′−C2′−C1′−H1′ torsional angle unconstrained. The H2′−C2′−C1′−H1′ torsional angle of 29.6° was obtained from the calculation, and this torsional angle corresponds to a 2′-^{3}H IE of 1.063, implying that 1.063 of 1.079 comes from positive hyperconjugation of σ(C2′−H2′) electrons to a partially empty $2p_z$ orbital and the rest comes from the effects described above.

Remote KIEs. Large normal intrinsic KIEs of 1.092 and 1.048 were measured for [Me-$^3H_3$]MTA and [5′-$^3H_2$]MTA, respectively. These isotope effects (Me-$^3H_3$ and 5′-$^3H_2$) arise from the net increase in the negative hyperconjugation, in the bound state, between the lone pairs (n_p) of sulfur and the σ^*-($C^{Me}H$) antibonding orbitals.[15a] In the unbound substrate, the methylthio group is free to rotate, canceling or reducing this hyperconjugation.

Transition-State Space. How well has this computational approach covered the possible geometric conformations of the transition state? The magnitude of primary (1′-^{14}C and 9-^{15}N) KIEs is only sensitive to the degree of dissociation of the C−N bond, the bond order to the nucleophile, and reaction coordinate motion at the transition state. Therefore, the geometry of the reaction coordinate (namely C1′−N9 and C1′−$O^{phosphate}$ distances) is a unique fit to the intrinsic KIE values. Ribose pucker is interpreted from the magnitude of the 2′-^{3}H KIE, which is proportional to the extent of hyperconjugation from σ(C2′−H2′) to the anomeric carbon. This isotope provides a unique conformation for the ribose pucker. The charge on the leaving group adenine is uniquely predicted by the magnitude of the 9-^{15}N KIE. Therefore, the geometry of the reaction coordinate, ribose pucker, and the ionization of the leaving group adenine are uniquely described by the intrinsic KIE values. The 5′-methio group can adopt multiple conformations to give the same KIE values. Therefore, computation alone is inadequate. Structural data from the crystal structure of human MTAP with the transition-state analogue, MT-ImmA, were used to position the 5′-methio group and therefore provide the origin of isotope effects for this specific geometry.

Conclusions

Human MTAP has a late dissociative S_N1 transition state, in which dissociation to the leaving group is complete and there is a significant bonding to the phosphate nucleophile. The formation of a ribosyl oxacarbenium ion is accompanied by the polarization or ionization of the 3′-OH by a phosphate, resulting in the formation of a 5-methylthioribosyl zwitterion at the transition state. The leaving group adenine is anionic with no bond order to the ribosyl zwitterion. The transition state of human MTAP therefore exists as a 5-methylthioribosyl zwitterion, where the positive charge on the anomeric carbon is stabilized by an anionic 3-oxygen as well as by a phosphate nucleophile, providing stabilization of the transition state. This is the first report to suggest the existence of a zwitterion−anion pair at an enzymatic transition state with the participation of a phosphate nucleophile, although the hydrolytic reaction of MTA catalyzed by *S. pneumoniae* MTAN has a similar ribosyl group at the transition state.

Many purine *N*-ribosyltransferases form transition states with neutral leaving groups and a cationic ribosyl group, thus generating a unit charge difference between the leaving group and the ribosyl group. Human MTAP also has a unit charge difference but generates it with a net neutral (zwitterionic) ribosyl group and an anionic purine leaving group. The cationic anomeric carbon is sandwiched between two structures with anionic character to facilitate the migration of the electrophilic center commonly seen in *N*-ribosyltransferases.[20]

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Supporting Information Available: Complete ref 7; frequencies of the unconstrained and constrained transition states. This material is available free of charge via the Internet at http://pubs.acs.org.

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