Published in final edited form as:

J Am Chem Soc. 2006 November 15; 128(45): 14691–14696. doi:10.1021/ja065419p.

Transition state structure of human 5'-methylthioadenosine phosphorylase†

Vipender Singh and Vern L. Schramm*

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

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'-³H], [1'-¹⁴C], [2'-³H], [4'-³H], [5'-³H], [9-¹⁵N] and [Me-³H₃] MTAs. The primary intrinsic KIEs (1'-¹⁴C and 9-¹⁵N) suggest that MTAP has a dissociative S_N1 transition state with cationic center at the anomeric carbon and insignificant bond order to the leaving group. The 9-¹⁵N intrinsic KIE of 1.039 also establishes an anionic character to the adenine leaving group, whereas the α-primary 1'-¹⁴C 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'-³H KIE is sensitive to the polarization of the 3'-OH group. Calculations suggest that a 4'-³H 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-³H₃] and [5'-³H] KIEs arise predominantly from the negative hyperconjugation of the lone pairs of sulphur 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.

Keywords

5'-methylthioadenosine; kinetic isotope effects; 5'-methylthioadenosine phosphorylase; polyamines; MTAP; transition state; zwitterionic transition state; transition state structure

Introduction

Kinetic isotope effects (KIE) 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 (³H, ¹⁴C and ¹⁵N) are substituted at positions expected to experience bond vibrational difference 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

†This work was supported by NIH Research Grant GM 41916.

*Corresponding author: Telephone (718) 430-2813, Fax (718) 430-8565, Email vern@aecom.yu.edu.

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-ribosyl transferases 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 *E. coli* 5'-methylthioadenosine nucleosidase (MTAN4) and Asp198 in bacterial purine nucleoside phosphorylase (PNP6)) 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 *S. 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 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'-methylthioribose1-phosphate (MTR-1-P) and adenine (Scheme 1). Disruption of MTAP has been shown to affect purine salvage, methionine and polyamine pathways and it has been proposed to be an anti-cancer drug target.⁷⁻¹⁰ This study suggests that human MTAP has a dissociative S_N1 transition state, and in this respect is similar to other members of the N-ribosyl transferase 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 pQE32 expression vector. The overexpressed MTAP, His₆ tagged at the N-terminus, was purified using Ni-NTA resin column using a 30-300 mM imadazole gradient. The purified protein was concentrated, dialyzed against 100 mM Tris, pH 7.9, 50 mM NaCl and 2 mM 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'-³H]MTA, [1'-¹⁴C]MTA, [2'-³H] MTA, [3'-³H]MTA, [4'-³H]MTA, [5'-³H]MTA, [methyl-³H₃]MTA and [8-¹⁴C]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 ^3H and ^{14}C labeled substrates with ^3H : ^{14}C in 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 pipettes). The remainder of the reaction mixture was allowed to react to completion and then applied to the column. Columns were washed with 2 volumes of 1 mM MTR and radioactive methylthioribose was eluted with 6 volumes of 15 mM MTR containing 50% ethanol. Each 1.0 mL of eluate was mixed with 9.0 mL scintillation fluid and counted for at least 3 cycles at 10 minutes per cycle. The ^3H to ^{14}C ratio was determined for partial and complete reactions and the KIEs were corrected to 0% hydrolysis by the equation:

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

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

Forward commitment factor

The isotope partition method¹² 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 of [8- ^{14}C]MTA containing 10^5 cpm was incubated for 10 seconds and then diluted with 180 μL of "Chase solution" containing a large excess of unlabeled MTA (2.6 mM) in 1 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 sec to allow a few turnovers and then quenched with 1N HCl. Product formation was measured by reverse phase HPLC using C-18 Deltapak column by 25% methanol and 50% of 100 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 intermediate using *ISOEFF 98* software.^{13a} All 3N-6 vibrational modes were used to calculate the isotope effects, but only those that exhibit shifts due to the isotopic substitution contribute to isotope effects. The isotope effects were calculated at the temperature of 298 K.

The applied geometric constraints were optimized iteratively to generate a transition state model for which the primary and the b-secondary EIEs closely match the intrinsic KIEs. The secondary 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 Supplementary Materials.

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} Based on 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 ¹⁵N9 (within experimental error of the theoretical maximum of 1.036 for complete dissociation of N-glycosidic bond^{15a}) and a large 1'-³H (the largest reported for any N-ribosyl transferases) preclude the existence of such equilibrium. Therefore the KIEs reported in Table 1 are intrinsic KIEs. The KIEs were measured for MTAs labeled at [1'-³H], [1'-¹⁴C], [2'-³H], [4'-³H], [5'-³H₂], [9-¹⁵N] and [Me-³H₃] using competitive conditions. 5'-¹⁴C MTA was used as a remote control for measuring tritium isotope effects and 5'-³H MTA was used for the same purpose for measuring 1'-¹⁴C and 9-¹⁵N/5'-¹⁴C KIE. The 1'-¹⁴C and 9-¹⁵N/5'-¹⁴C KIEs were corrected for the 5'-³H KIE. The measured KIEs were also corrected for the external forward commitment of 0.265 ± 0.027 (Figure 1) using Northrop's equation modified for irreversible reactions:

$$T(V/K) = \frac{T k + C_f}{1 + C_f}; \text{ Where } T(V/K) \text{ is an observed tritium isotope effect, } C_f \text{ is the forward commitment to catalysis. The intrinsic KIEs were obtained by correcting the observed KIEs (column 3, Table 1) for the forward commitment.}$$

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 both with 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 a S_N2 -like transition state, which is characterized by a large $1'-^{14}\text{C}$ KIE with significant bond order to the leaving group and the phosphate nucleophile. It had a single imaginary frequency of 295 i cm^{-1} (see Supplementary Information). The experimental intrinsic KIE of 1.029 for $1'-^{14}\text{C}$ MTA suggests that the anomeric carbon has a small but significant bond order to either to the leaving group or to an attacking phosphate nucleophile or to both at the transition state. The $1'-^{14}\text{C}$ KIE together with the $9-^{15}\text{N}$ intrinsic KIE of 1.039, which is consistent with the complete dissociation of the N-glycosidic bond at the transition state, suggests that the $1'-^{14}\text{C}$ KIE of 1.031 arises entirely from increased bonding to the oxygen of phosphate 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 $\text{O4}'\text{-C4}'\text{-C5}'\text{-S}$ and $\text{C4}'\text{-C5}'\text{-S}-\text{C}^{\text{me}}$ torsional angles. The calculations were performed by increasing the $\text{C1}'\text{-N9}$ distance in increments to $4.0\text{ \AA}$, 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 appearance of two imaginary frequencies (see Supplementary 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 with 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 ($1'-^{14}\text{C}$ and $9-^{15}\text{N}$) 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 $3\text{N}-6$ normal modes of the transition state model and the substrate were used to calculate equilibrium isotope effect (EIEs) using ISOEFF98.¹³ The applied external constraints were iteratively optimized until the calculated EIEs correlated with the intrinsic KIEs. At the transition state the oxygen of the phosphate nucleophile is $2.0\text{ \AA}$ from the anomeric carbon. The leaving group was modeled separately and is discussed below with $9-^{15}\text{N}$ KIE along with the properties of the transition state.

Intrinsic $9-^{15}\text{N}$, $1'-^{14}\text{C}$ and $1'-^3\text{H}$ KIEs

The $9-^{15}\text{N}$ 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 for the $9-^{15}\text{N}$ isotope effect of 1.036 calculated for the complete dissociation of N-glycosidic bond.¹⁵ Calculations on the adenine leaving group to study the effect of protonation at nitrogens N1, N3, N7 or N9 on the $9-^{15}\text{N}$ isotope effect shows that the protonation of N7 decreases the $9-^{15}\text{N}$ isotope effect from 1.036 to 1.025.¹⁵ Therefore an intrinsic KIE of 1.037 for $9-^{15}\text{N}$ 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 AMP 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 analog with protonated N7) shows equivalent O^{Asp220}-N7 distances within the crystallographic errors in these two structures (It is 3.0 Å in the MTA structure and 2.9 Å in the structure of MT-ImmA with human MTAP). However, the ionization of Asp220 is not revealed by crystallography and could make an important difference in binding energy of MTA and MT-ImmA.

The primary 1'-¹⁴C intrinsic KIE is the most useful probe for determining the mechanism of nucleophilic substitution reaction (S_N1 vs S_N2) of N-ribosyltransferases.¹⁷ A 1'-¹⁴C KIE of 1.00 to 1.030 indicates dissociative S_N1 transition states, 1.030 to 1.080 indicates significant associative interactions in S_N1 transition states and a KIE of greater than 1.080 indicates the properties of an S_N2 transition 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'-¹⁴C KIE indicates a change in hybridization at the anomeric carbon as it changes from sp^{2.83} hybridized in the substrate to sp^{2.40} at the transition state. These changes cause increased cationic character at the transition state (positive charge 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'-³H intrinsic KIE of 1.360 is consistent with the dissociative S_N1 transition state as indicated by the 1'-¹⁴C and 9-¹⁵N KIEs. The large 1'-³H 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'-³H KIE is also influenced by van der Waal interactions with active site residues and by the orientation of 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'-³H KIE.¹⁴ Although all these factors are difficult to model together, the large 1'-³H KIE is consistent with the dissociative transition state. Quantum mechanical tunneling is known to influence ³H-secondary kinetic isotope effects in hydride transfer reactions^{15b} but are 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'-³H KIE and evidence for a 5'-methylthioribosyl zwitterions

For dissociative S_N1 transition states, theoretical calculations predict a large inverse isotope for 4'-³H 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'-³H KIEs of 1.015 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 pairs of O4' to σ* (C4'-H4') antibonding orbital to increase to give a normal 4'-³H 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 normal intrinsic 4'-³H 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 is 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 $4'$ - ^3H 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 $4'$ - ^3H KIE to 1.047 in human MTAP compared to values of 1.015 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 $n_p(\text{O}4')$ to p -orbital ($\text{C}1'$) hyperconjugation to decrease and there is a corresponding increase in the $n_p(\text{O}4')$ to $\sigma^*(\text{C}4'-\text{H}4')$ 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 sulphate and the $2'$ -hydroxyl and the $3'$ -hydroxyl are 3.0 Å and 2.4 Å, respectively. These distances change to 2.8 Å 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 oxygen of $3'$ -hydroxyl and the nucleophile appears to increase and with a decrease in the O-O bond distance between $2'$ -hydroxyl and the phosphate nucleophile. The KIE analysis also supports the movement of a nucleophile towards 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 provide additional evidence concerning the importance of the $3'$ -hydroxyl in catalysis.¹⁹ The k_{cat}/K_m of human MTAP for MTA is $3.2 \times 10^6 \text{ M}^{-1} \text{ s}^{-1}$. It decreases to $9 \times 10^4 \text{ M}^{-1} \text{ s}^{-1}$ for $2'$ -deoxy MTA and to $10^3 \text{ M}^{-1} \text{ s}^{-1}$ for $3'$ -deoxy MTA. This change is predominantly a k_{cat} effect which decreases from 4.6 s^{-1} for MTA to 0.28 s^{-1} 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 $2'$ - ^3H KIE and ribosyl puckering

The positive hyperconjugation of $\sigma(\text{C}2'-\text{H}2')$ 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 $2'$ - ^3H KIE.^{15a} The contribution of this hyperconjugation to the total $2'$ - ^3H intrinsic KIE is dependent on the ribose puckering at the transition state. Its magnitude depends on the extent of overlap between the $\text{C}2'-\text{H}2'$ sigma bond and the $2p_z$ orbital. The magnitude of this effect varies as $\cos^2 \theta$ function of this overlap.^{15a} If the entire $2'$ - ^3H KIE originates from the hyperconjugative interaction between $\sigma(\text{C}2'-\text{H}2')$ and the p -orbital, an intrinsic $2'$ - ^3H KIE of 1.079 for human MTAP corresponds to the $\text{H}2'-\text{C}2'-\text{C}1'-\text{H}1'$ torsion angle of 33° and a small 3-endo pucker corresponding to the $\text{O}4'-\text{C}1'-\text{C}2'-\text{C}3'$ torsion angle of -13° . However, the $2'$ - ^3H KIE is also influenced by polarization of the $2'$ -OH and $3'$ -OH and rotation of $\text{H}2'-\text{C}2'-\text{O}-\text{H}$ torsional bond, whereas only positive hyperconjugation is influenced by puckering of the ribose.^{15a} To determine the fraction of the $2'$ - ^3H intrinsic KIE that comes exclusively from $\sigma(\text{C}2'-\text{H}2')$ 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 MTAP⁹) with human MTAP, and 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'-³H IE of 1.063, implying that 1.063 of 1.079 comes from positive hyperconjugation of σ (C2'-H2') electrons to 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-³H₃] MTA and [5'-³H₂] MTA, respectively. These isotope effects ([Me-³H₃] and [5'-³H₂]) arise from the net increase in the negative hyperconjugation, in the bound state, between the lone pairs (n_p) of sulphur 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'-¹⁴C and 9-¹⁵N) 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} distance) is a unique fit to the intrinsic KIE values. Ribose pucker is interpreted from the magnitude of the 2'-³H 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 9-¹⁵N 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. 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, was 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-ribosyl transferases form transition states with neutral leaving groups and a cationic ribosyl group, thus generating a unit charge difference between 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-ribosyl transferases.²⁰

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

References

1. Singh V, Lee JE, Nunez S, Howell LP, Schramm VL. *Biochemistry* 2005;44:11647–11659. [PubMed: 16128565]
2. Birck MR, Schramm VL. *J Am Chem Soc* 2004;126:2447–2453. [PubMed: 14982453]
3. Lewandowicz A, Schramm VL. *Biochemistry* 2004;43:1458–1468. [PubMed: 14769022]
4. Lee JE, Singh V, Evans GB, Tyler PC, Furneaux RH, Cornell KA, Riscoe MK, Schramm VL, Howell PL. *J Biol Chem* 2005;280:18274–18273. [PubMed: 15746096]
5. Singh V, Evans GB, Lenz DH, Painter GF, Tyler PC, Furneaux RH, Lee JE, Howell PL, Schramm VL. *J Biol Chem* 2005;280:18265–18273. [PubMed: 15749708]
6. Shi W, Basso LA, Santos DS, Tyler PC, Furneaux RH, Blanchard JS, Almo AC, Schramm VL. *Biochemistry* 2001;40:8204–8215. [PubMed: 11444966]
7. Frisch, MJ., et al. Gaussian03, Revision B.04. Gaussian, Inc.; Pittsburgh, PA: 2003.
8. Tabor CW, Tabor H. *Methods Enzymol* 1983;94:294–297. [PubMed: 6621389]
9. Singh V, Shi W, Evans GB, Tyler PC, Furneaux RH, Schramm VL. *Biochemistry* 2004;43:9. [PubMed: 14705926]
10. Harasawa H, Yamada Y, Kudoh M, Sugahara K, Soda H, Hirakata Y, Sasaki H, Ikeda S, Matsuo T, Tomonaga M, Nobori T, Kamihira S. *Leukemia* 2002;16:1799. [PubMed: 12200696]
11. Singh V, Shi W, Evans GB, Tyler PC, Furneaux RH, Schramm VL. *Biochemistry* 2004;43:9–18. [PubMed: 14705926]
12. Rose IA. *Methods Enzymol* 1980;64:47–59. [PubMed: 7374457]
- 13a. Anisimov V, Paneth P. *ISOEFF98. J Math Chem* 1999;26:75–86.
- 13b. Gawlita E, Lantz M, Paneth P, Bell AF, Tonge PJ, Anderson VE. *J Am Chem Soc* 2000;122:11660–11669.
14. Flükiger, P.; Lüthi, HP.; Portmann, S.; Weber, J. MOLEKEL 4.0. Swiss Center for Scientific Computing, Manno; Switzerland: 2000.
- 15a. Singh V, Schramm VL. *J Am Chem Soc.* submitted
- 15b. Pu J, Ma S, Garcia-Viloca M, Gao J, Truhlar DG, Kohen A. *J Am Chem Soc* 2005;127:14879–14886. [PubMed: 16231943]
- 15c. The computed transition state for human MTAP was solved without ionizing the ribosyl 3-hydroxyl group. Ionization of the 3-hydroxyl group forms a reactive 3-oxyanion which extracts a proton from the ribosyl 2-hydroxyl group in the in vacuo calculations and causes isotope effects unrelated to the enzymatic reaction coordinate. Experimental intrinsic KIEs, studies with substrate analogues, mutational and crystallographic studies do not support ionization or strong polarization of the 2-hydroxyl at the transition state. The effect of 3-hydroxyl polarization on the isotope effect pattern is discussed in the text and is expected to influence 2-³H, 3-³H and 4-³H IEs with largest IE expected at the 3-³H position. The effect of polarization on 2-³H and 4-³H IEs is discussed in the paper. The intrinsic 3-³H KIE has not yet been experimentally measured.
16. Parkin DW, Mentch F, Banks GA, Horenstein BA, Schramm VL. *Biochemistry* 1991;30:4586–4594. [PubMed: 2021651]
17. Berti PJ, Tanaka KSE. *Adv Phys Org Chem* 2002;37:239–314.
18. Appleby TC, Erion MD, Ealick SE. *Structure* 1999;7:629–641. [PubMed: 10404592]
19. Kung PP, Zehnder LR, Meng JJ, Kupchinsky SW, Skaltitzky D, Johnson MC, Maegley KA, Ekker A, Kuhn LA, Rose PW, Bloom LA. *Bioorg Med Chem Lett* 2005;48:2829–2833. [PubMed: 15911263]
20. Schramm VL, Shi W. *Curr Opin Struct Biol* 2001;11:657–665. [PubMed: 11751045]

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 as indicated by the arrow on the ordinate. 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 amount of labeled MTA on the active site before dilution with chase solution. The line is drawn from an ordinary least square fit of the data to the Michaelis-Menten equation. The intercept value is 0.21 and the forward commitment factor was calculated from the intercept using the expression: $(\text{intercept}/1-\text{intercept})$. 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/Å³. 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.

Scheme 1.

Phosphorolysis of MTA by human MTAP and the proposed transition state of the reaction. Atoms with differences in charge at the transition state are shown in blue. Details this transition state are presented in Table 2. KIE experiments used arsenate to reduce commitment factors.

Table 1KIEs measured at pH 7.5 for arsenolysis of MTA by human MTAP^a.

Substrate	Type of KIE	KIE ^b	Intrinsic KIEs ^d	Calculated KIEs
[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

^aExperimental KIEs are corrected to 0% substrate depletion.^bThe 1'-¹⁴C KIE was corrected for the 5'-³H KIE according to expression $\text{KIE} = \text{KIE}_{\text{observed}} \times 5'\text{-}^3\text{H KIE}$.^cThe 9-¹⁵N KIE was corrected for 5'-³H KIE according to expression $\text{KIE} = \text{KIE}_{\text{observed}} \times 5'\text{-}^3\text{H KIE}$.^dIntrinsic KIE values are corrected for the forward commitment shown in Figure 1.

Table 2

Geometric and electronic changes in representative models of the substrate and the transition state calculated using BILYP/6-31G** (human_MTAP).

Bond Type cont. (%)	Bond Length		Bond Order Change ^a	Hyperconjugation (kcal/mol) ^b				Orbital Changes ^c			
	GS	TS		Substrate		TS	$\Delta\Sigma$ (TS-GS)	GS hybrid	Carbon cont. (%)	TS hybrid	Carbon
				$\sigma \rightarrow$	$\rightarrow \sigma^*$						
C1'-H1'	1.0936	1.0848	-0.01584	10.95	8.10	7.12	6.97	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	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	sp ^{2.91}	62.45	sp ^{2.83}	63.98
C1'-N9	1.4582	NA	NA	8.84	28.64	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	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	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	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	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	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	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 (TS) (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 = ground state of substrate, TS = transition state. Lp1 is the sp-type lone pair; and Lp2 is p-type lone pair.

^d Lp2(C1');;

^e While Lp2(O4');;

^f Lp(O3');;

^g Lp2(S);;

^h Lp2(S);;

ⁱ Lp2(S);;

^j Lp2(S) are better acceptors in the substrate.

^k Lp2(O3') are better acceptors in the transition state;