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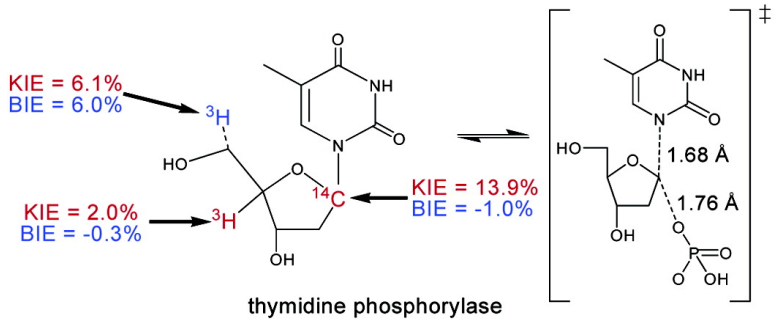

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Binding Causes the Remote [5′-^{3}H]Thymidine Kinetic Isotope Effect in Human Thymidine Phosphorylase

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Kinetic isotope effect (KIE) measurements provide the most direct route to the experimental characterization of enzymatic transition states. Competition between labeled and unlabeled substrate molecules provides a simple and direct method to measure isotope effects, but provides isotope effects on the parameter, *V/K*.[1−4] This collection of rate constants includes all steps between the free substrate up to and including the first irreversible step of the reaction (Figure 1).[1] Conditions can often be manipulated to make the catalytic step (k_5*) the first irreversible step, but little can be done to prevent previous steps, especially substrate binding, from contributing equilibrium binding isotope effects (BIEs) prior to formation of the transition state (TS). While these BIEs are usually assumed to be negligible in the interpretation of KIEs,[3] this is not always the case. BIEs of up to 8.5% have been measured for lactate dehydrogenase,[5] and 2−5% BIEs are not uncommon in those few systems where they have been measured.[5−8] It is possible to distinguish BIEs from *V/K* isotope effects if BIE and KIE are measured by independent methods.[5] Recently, we solved the transition-state structure for the reaction catalyzed by human thymidine phosphorylase (TP),[9] an enzyme of interest because of its link to aggressiveness and invasiveness of several types of human cancer.[10−17] The transition state was found to be a near-symmetric S_N2 nucleophilic displacement of thymine by inorganic phosphate (Figure 2).

The intrinsic *V/K* KIE for the remote secondary 5′-^{3}H position of thymidine was measured to be 6.1%.[9] No significant KIE is predicted for a position removed by four bonds from the site of reaction. It is almost 2-fold larger than that for bovine purine nucleoside phosphorylase (PNP, 3.3%) and is equivalent to that for human PNP (6.0%).[18,19] The PNP 5′-^{3}H KIE has been proposed to arise from the S_N1 nature of the PNP transition state. The oxacarbenium TS of PNP is proposed to be stabilized by placing the 5′-O lone-pair near 4′-O to alter its electron distribution.[20] In the case of TP, however, the TS has S_N2 character and precludes oxacarbenium ion formation.

Remote 5′-^{3}H KIEs can arise from 5′-dihedral immobilization upon thymidine (dT) binding, distortion of the 5′-sp^3 carbon geometry at the transition state, or a H-bond between enzyme and the 5′-OH. Binding of dT to the enzyme may cause some degree of negative charge buildup at the 5′-O, in turn affecting the bond lengths of the 5′-C−H bonds. Alternatively, the geometry of the 5′-O−H bond relative to the 5′-C−H bonds may become fixed upon binding. Model calculations suggest that a ∼2.6 Å H-bond between an active-site residue and the 5′-hydroxyl of thymidine or a dihedral angle of 0°−25° or 100°−115° between either proton and the hydroxyl could cause an observed isotope effect of approximately 6%.[7]

Competitive binding of [5′-^{14}C]dT and [5′-^{3}H]dT was measured in the presence of the phosphate analogue, SO_4^{2-} (Figure 1, $^3K_{ter}$). TP was found to catalyze the slow hydrolysis of dT in the absence of phosphate and the inclusion of 0.5 M sulfate eliminated

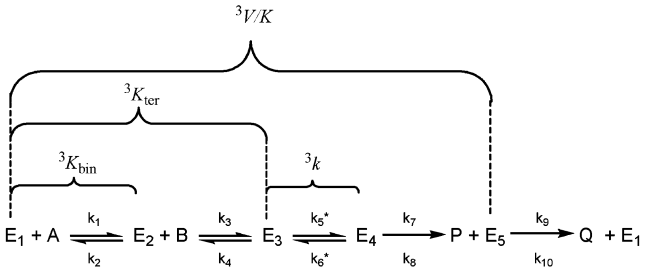

Figure 1. Graphical representation of the differences among binary BIE ($^3K_{bin}$), ternary BIE ($^3K_{ter}$), *V/K* KIE, and intrinsic KIE (^3k). The k_5* and k_6* steps represent the forward and reverse chemical reaction, respectively. Nomenclature conforms to that proposed by Northrop.[1] For simplicity, several steps included by Northrop have been omitted from this diagram.

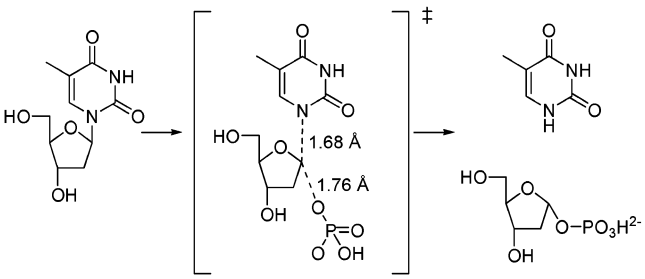

Figure 2. Reaction catalyzed by thymidine phosphorylase with the transition state features determined from KIE analysis.[9]

hydrolysis during the experiment. The presence of 0.5 M SO_4^{2-} might be considered to alter the hydrophobic fractionation factor for [5′-^{3}H]dT since tritium has a smaller vibrational volume than protium. However, quantitation of these effects is a fraction of 1% BIE per atomic substitution and similar to the error of the BIE of [5′-^{3}H]dT to TP.[21]

The BIEs were determined as described by Lewis and Schramm using the ultrafiltration method.[7,22] To a solution of 100 mM MES (pH 7.0), 500 mM ammonium sulfate and 3−8 μM [5′-*R*,*S*-mono-^{3}H]dT (radiolabel mixtures used are shown in Table 1), was added 5-8.5 μM TP to a final volume of 325 μL. Three 100 μM aliquots were removed and added to the upper wells of the ultrafiltration apparatus, and 22 psi N_2 was applied for 60−90 min or until approximately half of the solution had passed through the dialysis membrane (10−14 kDa molecular weight cutoff) into the lower well. From both upper and lower wells, 25 μL of solution was sampled (using a Hamilton syringe) and added to 1 mL of water in a scintillation vial. Ten milliliters of scintillation fluid (National Diagnostics Liquiscint) was added, and the samples were counted for at least 10 cycles of 10 min each.

Spectral deconvolution of ^{3}H and ^{14}C was performed using a ^{14}C standard in a matrix identical to that of the BIE samples.[9] The BIE was calculated as the quotient of the ratio of ^{14}C to ^{3}H bound

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Table 1. Radiolabeled Substrates, BIEs, and KIEs for TP

label	substrates[a]	BIE[b]	KIE[a]
5′-^{3}H	[5′-^{3}H]dT; [5′-^{14}C]dT	1.060 ± 0.002 (5)	1.061 ± 0.003
4′-^{3}H	[4′-^{3}H]dT; [5′-^{14}C]dT	0.997 ± 0.005 (3)	1.020 ± 0.003
1′-^{14}C	[1′-^{14}C]dT; [4′-^{3}H]dT	0.990 ± 0.004 (3)	1.139 ± 0.005

[a] Substrate synthesis and KIEs are detailed in ref 9. [b] BIE ± standard deviation. The number of replicates for each experiment is given in parentheses.

to the enzyme (R_b) and free in solution (R_f) according to eq 1:

$$R_b = \left(\frac{1}{1-f}\right)R_m - \left(\frac{f}{1-f}\right)R_f \quad (1)$$

The fraction of substrate unbound in the upper well (f), is the ratio of ^{3}H below the membrane to that above, R_m is the ^{14}C to ^{3}H ratio above the membrane, R_f is the ratio below the membrane, and R_b is the isotopic ratio of bound substrate. Although TP concentration increases throughout the experiment, neither the [E·dT]/[E] ratio nor $[dT]_{free}$ changes with ultrafiltration; thus, BIE is independent of concentration.

The measurement of bound/free dT also provides a measure for the dissociation constant of dT from the TP·SO_4·dT complex. For the conditions used here, the K_d was 70 ± 12 μM, consistent with the K_m value of 54 ± 9 μM from steady-state kinetic analysis with PO_4^{2-}. Forward commitment was insignificant in the KIE experiments, predicting that K_m and K_d should be equivalent. However, K_d is in the presence of sulfate, and K_m is in the presence of phosphate. The similarity in the values suggests that SO_4^{2-} provides a similar environment for dT binding.

The 6.0% BIE exhibited at the 5′ position accounts, within error, for the entire 6.1% KIE previously reported (Table 1). This equilibrium isotope effect explains that the KIE measured at this remote position is introduced upon the formation of the Michaelis complex and is conserved to the TS. Thus, the 5′-^{3}H KIE is an equilibrium binding interaction maintained through the rate-limiting TS.

In addition to the 5′-^{3}H BIE, the 4′-^{3}H and 1′-^{14}C BIEs were measured to distinguish BIEs from KIEs. The 4′-^{3}H BIE is unity, within error. Therefore, the intrinsic 4′-^{3}H KIE of 1.020 is a direct result of the geometry at the transition state, rather than binding interactions. The 4′-CH proton is remote from most interactions which could cause a BIE, and the 4′-CH is unlikely to be engaged in a H-bond. The lack of a 4′-^{3}H BIE also suggests that the enzyme does not substantially alter the conformation of the ribosyl ring upon thymidine binding. Thus, ground-state destabilization via modification of the deoxyribose ring geometry is unlikely to play a significant part in catalytic rate enhancement.

The most diagnostic KIE for transition-state analysis of TP is the large primary 1′-^{14}C KIE of 1.139. Binding is not expected to cause a ^{14}C KIE at this or other positions since reaction coordinate motion and rehybridization dominate ^{14}C KIEs. Measurement of the 1′-^{14}C BIE confirms that the measured 1′-^{14}C arises directly from electronic and geometric changes at the transition state that did not exist in the Michaelis complex. This eliminates any possible equilibrium binding component to the reported primary 1′-^{14}C KIE.

An X-ray crystal structure of human TP with a bound inhibitor (5-chloro-6-[1-(2-iminopyrrolidinyl)methyl]uracil hydrochloride, TPI) has been reported.[23] However, the deoxyribose binding pocket is not defined since the iminopyrrolidine moiety of TPI has no 5′-OH group. A computationally predicted dT binding site does not establish interactions for 5′-OH group,[24] but suggests that the 5′-OH of the ribosyl moiety may bind to active-site residues H116, S117, or T118, as judged from the structure of Norman et al.[23] with dT superimposed on TPI. An interaction between the 5′-OH and the 2-O of the thymine ring is also possible. Structures of human TP containing thymidine or thymidine analogue are required to solve this interaction. In *Lactobacillus casei* TP, 5′-deoxythymidine was 42% as active as thymidine,[25] suggesting dihedral freezing and not catalytic participation.

Remote KIEs at the 5′-position of nucleosides and nucleotides are common in *N*-ribosyl transferases.[9,18,19,22] In enzymes with ribooxacarbenium TSs, 5′-OH neighboring group participation has been seen crystallographically and is consistent with electronic effects. However, TP is known to form an S_N2 TS, and 5′-OH participation would not assist TS formation.[9] Glucose binding studies to hexokinase have established that dihedral freezing without electronic contribution to TS stabilization can give rise to isotope effects as large as the 6% BIE seen for [5′-^{3}H]dT to TP. This KIE in TP therefore originates from equilibrium binding effects which persist through the TS.

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