

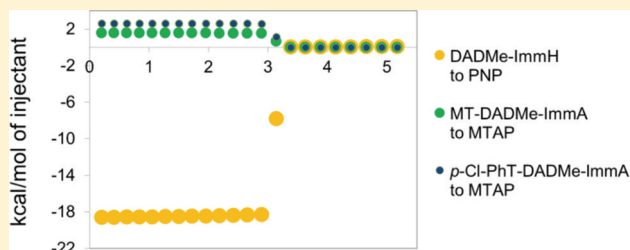
Entropy-Driven Binding of Picomolar Transition State Analogue Inhibitors to Human 5'-Methylthioadenosine Phosphorylase

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ABSTRACT: Human 5'-methylthioadenosine phosphorylase (MTAP) links the polyamine biosynthetic and S-adenosyl-L-methionine salvage pathways and is a target for anticancer drugs. *p*-Cl-PhT-DADMe-ImmA is a 10 pM, slow-onset tight-binding transition state analogue inhibitor of the enzyme. Titration of homotrimeric MTAP with this inhibitor established equivalent binding and independent catalytic function of the three catalytic sites. Thermodynamic analysis of MTAP with tight-binding inhibitors revealed entropic-driven interactions with small enthalpic penalties. A large negative heat capacity change of -600 cal/(mol K) upon inhibitor binding to MTAP is consistent with altered hydrophobic interactions and release of water. Crystal structures of apo MTAP and MTAP in complex with *p*-Cl-PhT-DADMe-ImmA were determined at 1.9 and 2.0 Å resolution, respectively. Inhibitor binding caused condensation of the enzyme active site, reorganization at the trimer interfaces, the release of water from the active sites and subunit interfaces, and compaction of the trimeric structure. These structural changes cause the entropy-favored binding of transition state analogues. Homotrimeric human MTAP is contrasted to the structurally related homotrimeric human purine nucleoside phosphorylase. *p*-Cl-PhT-DADMe-ImmA binding to MTAP involves a favorable entropy term of -17.6 kcal/mol with unfavorable enthalpy of 2.6 kcal/mol. In contrast, binding of an 8.5 pM transition state analogue to human PNP has been shown to exhibit the opposite behavior, with an unfavorable entropy term of 3.5 kcal/mol and a favorable enthalpy of -18.6 kcal/mol. Transition state analogue interactions reflect protein architecture near the transition state, and the profound thermodynamic differences for MTAP and PNP suggest dramatic differences in contributions to catalysis from protein architecture.



5'-Methylthioadenosine (MTA) is produced from decarboxy-SAM by spermidine and spermine synthases, sequential enzymes in the polyamine biosynthesis pathway.¹ In humans, the metabolism of MTA relies solely on MTA phosphorylase (MTAP) to catalyze the phosphorolysis of MTA to adenine and 5-methylthio- α -D-ribose 1-phosphate (MTR-1-P).^{2,3} These products can be utilized for SAM resynthesis. Thus, MTAP links polyamine biosynthesis and SAM salvage. Polyamine biosynthesis is required for rapid cell proliferation.⁴ The accumulation of MTA can cause feedback inhibition of polyamine production and recycling of MTA to SAM. These features suggested MTAP inhibition as an approach for the development of anticancer drugs.^{5,6} The inhibition of MTAP may also affect SAM-related protein and DNA methylation and thus cause antiproliferative responses in cancer cells.⁶

Human MTAP is a structural homologue of human purine nucleoside phosphorylase (PNP), but PNP prefers 6-oxo-purine nucleoside substrates. Although these enzymes share only 27% sequence identity, they share similar active sites and overall topology.⁷ Both enzymes are homotrimers with three active sites located near the subunit interfaces. Their active sites

contain three binding regions corresponding to the purine-, (methylthio)ribose-, and phosphate-binding sites.

The transition state structure for human MTAP has been determined by kinetic isotope effects and computational modeling.⁸ MTAP forms a late dissociative transition state, featuring complete dissociation of the adenine leaving group with significant participation of the phosphate nucleophile (Figure 1A). The ribocation transition state is stabilized by the 3'-oxygen and phosphate, forming a 5-methylthioribosyl zwitterion at the transition state.⁸

Two generations of transition state analogue inhibitors have been synthesized for human MTAP (Figure 1B). MT-ImmA (K_i of 1 nM) is a first-generation inhibitor, resembling an early transition state with ribocation character and an elevated pK_a at N7 of the leaving group.⁹ MT-DADMe-ImmA (K_i of 90 pM) is a second-generation inhibitor and mimics the fully dissociative transition state of MTAP by increasing the distance between deazaadenine and the pyrrolidine group through a methylene

Received: August 19, 2011

Revised: October 7, 2011

Published: October 10, 2011



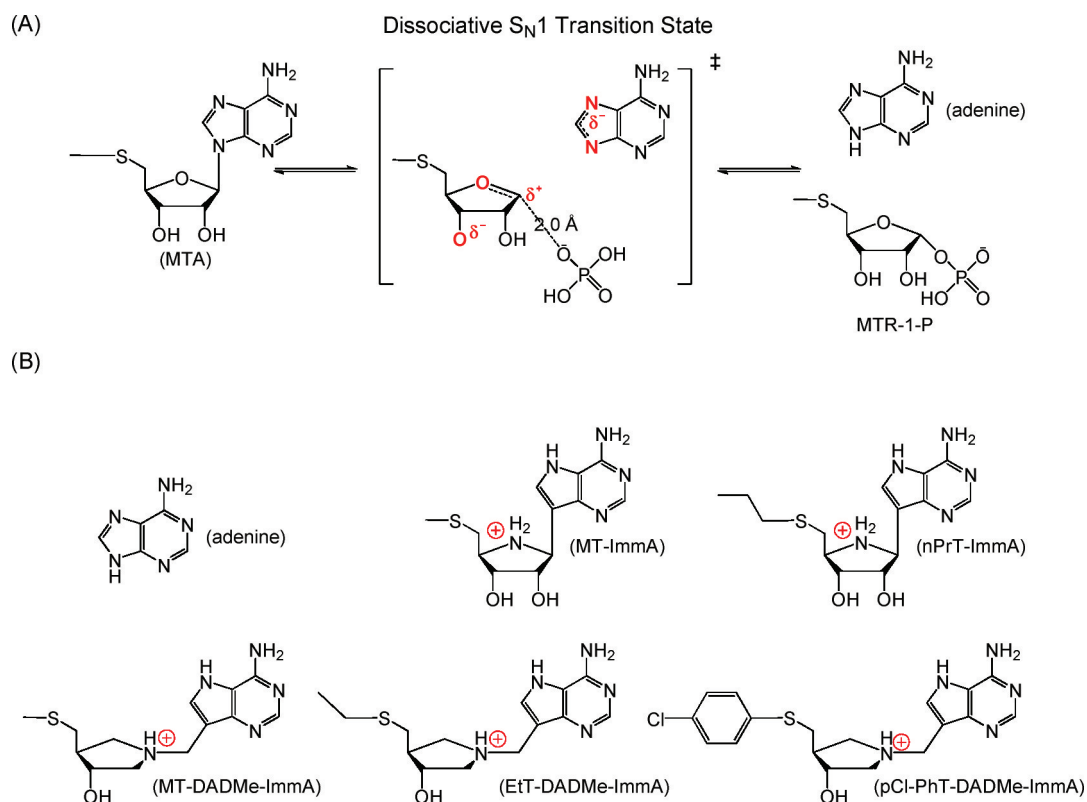


Figure 1. (A) Transition state of human MTAP. Atoms in red are those with charge differences at the transition state. (B) Adenine and iminoribitol-based (upper row) or hydroxypyrrolidine-based (lower row) transition state analogue inhibitors of MTAP.

bridge.¹⁰ MT-DADMe-ImmA induces apoptosis in two head and neck carcinoma cell lines (FaDu and Cal27) and prevents the growth of FaDu tumors in a mouse model.⁶ MT-DADMe-ImmA also inhibits the tumor growth and metastases of human lung cancer cell lines in mouse xenografts.¹¹ Its broad anticancer activity with low systemic toxicity makes MT-DADMe-ImmA a candidate for anticancer activity.

Thermodynamic parameters for the binding of picomolar transition state analogue inhibitors to human PNP revealed large favorable enthalpy opposed by an unfavorable entropy term.¹² This pattern is expected by the general nature of enthalpy–entropy compensation, where the transition state analogue binding causes the protein to become stabilized and organized through favorable hydrogen bond and ion-pair contacts with the inhibitor. We wanted to investigate if this is a general phenomenon by examining the related MTAP system.

Binding studies with MTAP involve the thermodynamics of ligand binding using the product adenine and two generations of transition state analogue inhibitors. The results are unprecedented, revealing entropy-driven binding for adenine and for transition state analogue interactions with MTAP. The crystal structures and hydrodynamic properties of apo and liganded MTAP are described and compared to the binding thermodynamics found with human PNP. Thermodynamic properties and subunit cooperativity of MTAP are both distinct from the related human PNP. The work highlights the diversity of enzymatic function and the interaction of transition state analogues.

MATERIALS AND METHODS

Chemicals. Transition state analogue inhibitors of human MTAP were synthesized and provided by Dr. Gary B. Evans

and colleagues of the Carbohydrate Chemistry Team, Industrial Research Ltd. These inhibitors include two first-generation ImmAs (MT-ImmA and PrT-ImmA) and three second-generation ImmAs (MT-DADMe-ImmA, EtT-DADMe-ImmA, and *p*-Cl-PhT-DADMe-ImmA) (Figure 1B). Their synthesis and characterization are detailed elsewhere.^{10,13} Adenine was purchased from Sigma. Other chemicals and reagents were obtained from Sigma or Fisher Scientific and were of highest grade available, unless otherwise specified.

Plasmid Construction. A synthetic gene for human MTAP was designed from the NCBI reference sequence NP_002442.2 and was synthesized and cloned into the pJexpress 414 expression vector by DNA2.0 Inc. The recombinant human MTAP has 14 additional amino acids at N-terminus of the native enzyme, including a His₆ tag and a TEV protease cleavage site.

Enzyme Purification and Preparation. The plasmid containing the synthetic MTAP gene was transformed into BL21-CodonPlus(DE3)-RIPL *E. coli* cell and was grown overnight at 37 °C in 25 mL of LB medium containing 100 μ g/mL ampicillin. This culture was transferred into 1 L of LB containing ampicillin medium, and the cell growth was continued at 37 °C until the OD₆₀₀ reached 0.7. Protein expression was induced by adding IPTG to 1 mM final concentration. After 4 h induction at 37 °C, the cells were harvested by centrifugation at 4500g for 30 min. Cell pellet was suspended in 20 mL of lysis buffer (15 mM imidazole, 300 mM NaCl, and 50 mM phosphate, pH 8.0), with EDTA-free protease inhibitor (2 tablets) added according to supplier recommendations (from Roche Diagnostics) and lysozyme (20 mg) added to the slurry. Cells were disrupted twice with a French Press and centrifuged at 20000g for 30 min. The supernatant was loaded onto a 3 mL column of Ni-NTA Superflow resin pre-equilibrated with 15 mL

of lysis buffer. The column was washed with 15 mL of wash buffer (80 mM imidazole, 300 mM NaCl, and 50 mM phosphate, pH 8.0), and enzyme was eluted with 12 mL of elution buffer (250 mM imidazole, 300 mM NaCl, and 50 mM phosphate, pH 8.0). Purified enzyme was dialyzed against 100 mM phosphate, pH 7.4, with 2 mM DTT and concentrated to about 5 mg/mL. Enzyme was stored frozen at -80°C and had a purity of >95% on SDS-PAGE. The extinction coefficient of this recombinant human MTAP is estimated to be $31.4\text{ mM}^{-1}\text{ cm}^{-1}$ at 280 nm (ProtParam program from ExPASy). The k_{cat} and K_{MTA} values of purified MTAP are similar to previously reported data,¹⁴ indicating that this enzyme is catalytically equivalent to native human MTAP.

Purified enzyme had approximately two-thirds of its sites occupied by tightly bound adenine, consistent with a previous report of a MTAP structure containing an adenine complex.⁷ Co-purified adenine was identified by (a) denaturing the enzyme using perchloric acid (final content of 10% v/v), (b) obtaining the supernatant by centrifugation, (c) performing a UV-vis scan of the supernatant (220–350 nm), and (d) calculating the concentration of adenine using ϵ_{260} of $13.0\text{ mM}^{-1}\text{ cm}^{-1}$. Adenine copurifying with MTAP was removed by dialyzing purified enzyme against 100 mM phosphate, pH 7.4, containing 0.5% (v/v) charcoal for 3 h or by extensive dialysis of the enzyme against 100 mM phosphate, pH 7.4, with multiple dialysate changes. Phosphate at 100 mM, the condition used here for binding experiments, weakens adenine binding.

Enzymatic Assays. Adenine formation was coupled to the formation of 2,8-dihydroxyadenine by xanthine oxidase.¹⁵ The extinction coefficient of this coupled assay was $15.5\text{ mM}^{-1}\text{ cm}^{-1}$ at 305 nm.¹⁶ Reactions were carried out at 25°C in 1 cm path length cuvettes with a final volume of 1 mL. Reaction mixtures contained 100 mM phosphate, pH 7.4, 200 μM MTA, 1 mM DTT, 1 unit of xanthine oxidase, and appropriate amounts of MTAP.

A direct assay involves the absorbance decrease at 275 nm on conversion of MTA to adenine, resulting in an extinction coefficient of $1.6\text{ mM}^{-1}\text{ cm}^{-1}$. Reactions were carried out at 25°C in 1 cm path length cuvettes with a final volume of 1 mL. Reaction mixtures contained 100 mM potassium phosphate, pH 7.4, 50 mM Hepes, pH 7.4, 150 μM MTA, and appropriate amounts of MTAP. Reactions were initiated by addition of enzyme, and the initial rates were monitored using a Cary 300 UV-vis spectrophotometer. Control rates (no MTAP) were subtracted from initial rates for all reactions.

Catalytic Site Titration of Human MTAP. Human MTAP was titrated with *p*-Cl-PhT-DADMe-ImmA. Titrations were monitored using residual enzyme activity and by fluorescence spectroscopy. Enzyme was prepared in Eppendorf tubes followed by the addition of various concentrations of inhibitor to each tube. Enzyme-inhibitor mixtures were incubated at 25°C for an hour and assayed. Assays were as described in “enzymatic assays” except that reactions were initiated by adding enzyme-inhibitor mixtures. Initial rates of the reactions were plotted as a function of the ratio of inhibitor concentration to MTAP trimer concentration. The titration mixtures (1 μM enzyme and various concentration of inhibitor) were excited at 298 nm when monitored by fluorescence spectroscopy. The slit width set to 3 for excitation and 2 for emission. Excitation spectra were collected from 310 to 450 nm, and the relative intensities at 350 nm were plotted

with the ratio of inhibitor concentration to MTAP trimer concentration.

Isothermal Titration Calorimetry Studies. ITC studies were carried out using a VP-ITC MicroCalorimeter. Adenine that purified with MTAP was removed using the methods mentioned above. Apo protein was dialyzed overnight against 100 mM potassium phosphate buffer, pH 7.4. Dialysate and apo protein were filtered (Millipore, $0.2\text{ }\mu\text{m}$). Inhibitor solutions were prepared by dissolving the inhibitor into filtered dialysate. Apo protein sample and inhibitor solution were degassed (Microcal Thermovac) for 15 min and were loaded into a 1.46 mL sample cell and 250 μL injection syringe. The final concentration prior to titrations was 40 μM protein and 600 μM inhibitor. Titration of human MTAP used 30 injections of 6–8 μL with 4 min between injections. ITC data of first-generation inhibitors were fitted to a model of two distinct independent sites. Data of second-generation inhibitors were fitted to a model for a single set of identical sites. Data for adenine binding were fitted to a model for three sequential binding sites. MTAP inhibitors were also titrated into buffer to correct for heat of dilution. Those controls were subtracted from the corresponding ITC data using the “subtract reference” function in Origin 7.0. MTAP samples with or without reducing reagent (2 mM β -mercaptoethanol) resulted in identical ITC data. Enzyme samples prepared by either adenine removal method gave the same results in ITC studies.

Data Processing of ITC Studies. ΔG values were calculated from K_i using eq 1. The K_i of ImmAs was reported previously, given by equilibrium dissociation constant following slow-onset inhibition.^a The K_i values of adenine were determined by ITC titration. The entropy terms ($-T\Delta S$) were calculated from eq 2 (the Gibbs free energy expression).

$$\Delta G = RT \ln K_i \quad (1)$$

$$\Delta G = \Delta H - T\Delta S \quad (2)$$

Equations 3, 4, and 5 represent the total heat content Q of the solution contained in V_0 (active cell volume) for a single set of identical sites, two sets of independent sites, and sequential binding sites models, respectively. Equation 6 represents the heat released, ΔQ_i , from the i th injection for those three models. In eqs 3–6, n is the number of sites, Θ is the fraction of sites occupied by inhibitor, F_i is the fraction of total protein sample having i bound inhibitors, dV_i is the injection volume, and ΔH is the molar heat of inhibitor binding. Data fitting follows the same procedures for all three models. Initial guesses of n , K_a (association constant of inhibitor, the reciprocal of equilibrium dissociation constant K_d), and ΔH were made by the Origin 7.0 software, followed by the calculation of ΔQ_i for each injection. ΔQ_i data were then compared with the corresponding heat measured for each injection. Optimizations of the values of n , K_a , and ΔH were achieved using standard Marquardt methods and were iterated until there was no further significant improvement of data fit. The known K_a values can be directly input into Origin 7.0 for more accurate fitting. This and more detailed information related to data fitting are available from the User's Manual for the VP-ITC MicroCalorimeter.

$$Q = n\Theta M_t \Delta H V_0 \quad (3)$$

$$Q = M_t V_0 (n_1 \Theta_1 \Delta H_1 + n_2 \Theta_2 \Delta H_2) \quad (4)$$

$$Q = M_i V_0 (F_1 \Delta H_1 + F_2 [\Delta H_1 + \Delta H_2] + F_3 [\Delta H_1 + \Delta H_2 + \Delta H_3]) \quad (5)$$

$$\Delta Q_i = Q_i + \frac{dV_i}{V_0} \left(\frac{Q_i + Q_{i-1}}{2} \right) - Q_{i-1} \quad (6)$$

Determination of Heat Capacity Change. ITC studies were carried out at different temperatures (15, 20, 25, 30, and 37 °C) to determine the heat capacity change (ΔC_p) of MT-ImmA binding to MTAP. The ΔH values were plotted as a function of temperature and exhibited a linear pattern. The slope of the line indicates the heat capacity change upon ligand binding.¹⁷

Crystallization and Structure Determination of Apo MTAP and MTAP:Inhibitor Complex. Apo MTAP crystals were obtained by sitting-drop vapor diffusion by mixing recombinant MTAP (13 mg/mL) in 20 mM phosphate pH 7.4, 1 mM DTT with reservoir buffer composed of 0.2 M NaCl, 20% PEG3000, and 0.1 M Hepes pH 7.5 at 18 °C. MTAP:inhibitor complexes were formed by incubating MTAP (19 mg/mL) in 20 mM Tris pH 7.4 with 1 mM *p*-Cl-PhT-DADMe-ImmA and 6 mM potassium phosphate pH 7.4 on ice. The MTAP:*p*-Cl-PhT-DADMe-ImmA:phosphate complex was cocrystallized by hanging drop or sitting drop vapor diffusion methods with precipitant composed of 0.2 M NaCl, 10% PEG8000, and 0.1 M Na/K phosphate pH 6.2 at 18 °C. Crystals were transferred to the mother liquor supplemented with 20% glycerol and flash frozen in liquid N₂ before data collection. X-ray diffraction data were collected at the X29 beamline of Brookhaven National Laboratory on an ADSC Q315 detector at 100 K. The data were processed using the HKL2000 program suite,¹⁸ and data reduction is summarized in Table 1.

The structures of apo MTAP and its inhibitor complexes were determined by molecular replacement using the crystal structure of adenine-omit MTAP (PDB ID: 1CB0) as the search model using the program Molrep.^{7,19} Models without inhibitor were iteratively rebuilt in COOT and refined in Refmac5.^{20,21} Manual inhibitor building was initiated only after the R_{free} decreased below 30% and was guided by clear ligand density in $F_o - F_c$ electron density maps contoured at 3σ . Data processing and refinement statistics are summarized in Table 1.

Analytical Ultracentrifugation Experiments. Sedimentation velocity experiments were conducted and analyzed as described previously²² at a MTAP concentration of 4.5 mg/mL (134 μ M) and inhibitor concentrations of 400 μ M. Of the several hundred interference scans acquired for each sample, the software DCDT+ (version 2.3.2) selected ~80 scans for the time derivative calculation.

RESULTS AND DISCUSSION

Titration of Human MTAP. The stoichiometry of inhibitor binding to the MTAP trimer was probed by titration with *p*-Cl-PhT-DADMe-ImmA, a potent 10 pM inhibitor of MTAP.⁵ The titration was monitored by enzyme activity assays and fluorescence spectroscopy. The activity of enzyme–inhibitor mixtures was a linear function of inhibitor concentration with a slope indicating three equivalent, independent active sites filling with inhibitor. Thus, the three active sites of the MTAP trimer exhibit equal catalytic activities and comparable binding affinities for the transition state

Table 1. Data Collection and Refinement Statistics

	<i>p</i> -Cl-PhT-DADMe-ImmA/PO ₄ (PDB ID 3OZC)	PO ₄ (PDB ID 3OZE)
<i>data collection</i>		
space group	P321	P2 ₁
cell dimensions		
<i>a</i> , <i>b</i> , <i>c</i> (Å)	123.3, 123.3, 44.9	79.5, 81.8, 130.3
α , β , γ (deg)	90.0, 90.0, 90.0	90.0, 91.0, 90.0
resolutions ^a (Å)	50.00–1.93 (2.00–1.93)	20.00–2.00 (2.07–2.00)
R_{sym} (%)	7.7 (37.6)	7.3 (67.2)
$I/\sigma I$	24.7 (4.7)	14.9 (1.6)
completeness (%)	99.5 (94.8)	99.9 (97.6)
redundancy	7.2 (7.0)	4.0 (3.9)
<i>refinement</i>		
resolution (Å)	50.00–1.93	20.00–2.00
no. unique reflections	29533	111658
$R_{\text{work}}/R_{\text{free}}$ (%)	13.1/15.4	21.5/26.1
<i>B</i> -factors (Å ²)		
protein (main chain)	20.4	50.0
protein (side chain)	22.8	51.7
water	22.7	45.8
ligand	19.5	40.6
no. of atoms		
protein	2112	12241
water	67	270
ligand	31	30
rms deviations		
bond lengths (Å)	0.013	0.015
bond angles (deg)	1.46	1.48
<i>Ramachran analysis</i>		
favoured region (%)	98.2	97.8
allowed region (%)	1.8	2.2
disallowed region (%)	0	0
coordinate error by Luzzati plot (Å)	0.14	0.34

^aNumbers in parentheses are for the highest-resolution shell. One crystal was used for each data set.

analogue inhibitor (Figure 2). Relative fluorescence intensities plotted as a function of the molar ratio of inhibitor to human

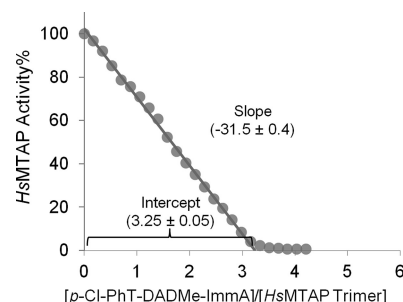


Figure 2. Titration of *p*-Cl-PhT-DADMe-ImmA to human MTAP. MTAP (200 nM) was incubated with inhibitor in 100 mM potassium phosphate, pH 7.4, to achieve equilibrium and assayed for MTAP activity.

MTAP trimer also gave a linear pattern indicating equivalent conformational changes to the subunits as inhibitor binds (not shown). Thus, trimeric MTAP appears to contain three independent functional catalytic sites.

Table 2. Thermodynamics for Binding Adenine and Transition State Analogue Inhibitors to Human MTAP

inhibitor	K_d (nM) [n] ^a	ΔG (kcal/mol)	ΔH (kcal/mol)	$-T\Delta S$ (kcal/mol)
adenine	3000 ± 1000^b [1]	-7.6 ± 0.2	-0.003 ± 0.2	-7.6 ± 0.3
	2100 ± 80^b [1]	-7.7 ± 0.2	2.4 ± 0.2	-10.1 ± 0.3
	80000 ± 80000^b [1]	-5.6 ± 0.5	-2.6 ± 0.8	-3.0 ± 0.9
MT-ImmA	1.0 ± 0.1^c [2.42 ± 0.04]	-12.3 ± 0.1	0.24 ± 0.04	-12.5 ± 0.1
	700 ± 300^b [0.58 ± 0.09]	-8.4 ± 0.3	2.7 ± 0.4	-11.1 ± 0.5
nPrT-ImmA	0.214 ± 0.01^c [2.13 ± 0.04]	-13.2 ± 0.03	0.24 ± 0.03	-13.4 ± 0.1
	900 ± 600^b [0.9 ± 0.1]	-8.2 ± 0.4	1.1 ± 0.2	-9.3 ± 0.4
MT-DADMe-ImmA	0.09 ± 0.01^d [3]	-13.7 ± 0.1	1.5 ± 0.1	-15.2 ± 0.1
EtT-DADMe-ImmA	0.034 ± 0.003^d [3]	-14.3 ± 0.1	1.8 ± 0.1	-16.1 ± 0.1
p-Cl-PhT-DADMe-ImmA	0.010 ± 0.005^d [3]	-15.0 ± 0.3	2.6 ± 0.1	-17.6 ± 0.3

^a n is the number of sites. ^b K_d values are derived from ITC data fitting. ^cFrom ref 9. ^dFrom ref 5.

Transition state analogue binding to MTAP differs remarkably from that of human PNP despite their trimeric structural similarity and their common chemistry of purine nucleoside phosphorolysis. Binding a transition state analogue inhibitor to a single site of trimeric human PNP causes complete inhibition indicating a one-third-the-sites functional interaction.²³ Human PNP is thus proposed to have a sequential catalytic action at each subunit, reminiscent of other trimeric proteins with catalytic function, including F_1 ATPase. Binding of transition state analogues to human PNP demonstrates strong negative cooperativity, with pM, nM, and μ M binding at the first, second, and third sites, respectively.²³

Isothermal Titration Calorimetry Studies. MTAP isothermal titration calorimetry with five transition state analogue inhibitors included first-generation analogues (MT-ImmA, nPrT-ImmA) and second-generation analogues (MT-DADMe-ImmA, EtT-DADMe-ImmA, and p-Cl-PhT-DADMe-ImmA). Accurate K_d (equilibrium dissociation constant) values for these inhibitors were obtained by slow-onset competitive kinetic inhibition assays^{5,9} and used for ITC data processing (Table 2 and Figure 3).

ITC Profiles for Binding First-Generation MTAP Inhibitors. First-generation MTAP inhibitors bind with small enthalpic heat uptakes in a cooperative manner to the three sites of trimeric MTAP (Table 2 and Figure 3B). Thermodynamic changes are similar for binding at the first two sites, and a larger change occurs in the same direction as the third site is filled (Figure 3B). MT-ImmA binding to the first two sites gives K_d values of 1 and 700 nM for the third site. As the steady-state kinetic K_i value for MT-ImmA is 1 nM, and complete inhibition is observed,⁵ the result suggests a functional cooperativity with substrate and MT-ImmA binding so that at near saturating MTA substrate, as occurs in steady-state assays, the three sites show similar affinity for the inhibitor. Binding to the third site shows a greater thermal change, also in the direction of heat uptake by the complex.

Binding of nPrT-ImmA to MTAP gave a thermal profile similar to that for MT-ImmA with calculated K_d values of 214 pM for the first two sites and \sim 900 pM for the third site. Catalytic site negative cooperativity in the energy landscape is apparent from the nonlinear response to catalytic site filling with greater thermal energy uptake for filling of the third site for both of the first-generation MTAP inhibitors (Figure 3B). As with MT-ImmA, these cooperative differences are masked in steady-state kinetic analysis of K_i values by the near-saturating MTA in these assays.

Binding of first-generation ImmA inhibitors is completely dominated by favorable entropic terms with small enthalpic

penalties. The ΔG , ΔH , and $-T\Delta S$ values are -12.3 , 0.2 , and -12.5 kcal/mol, respectively, for the major binding sites of MT-ImmA. The binding ΔG , ΔH , and $-T\Delta S$ values are -13.2 , 0.2 , and -13.4 kcal/mol, respectively, for the major binding sites of nPrT-ImmA.

Binding of Second-Generation MTAP Inhibitors. Titration of MTAP catalytic function with p-Cl-PhT-DADMe-ImmA indicated three catalytically functional and independent binding sites (Figure 2A), and these are known by inhibition kinetics to have dissociation constants of 10 pM.¹⁰ ITC data with three second-generation MTAP inhibitors (MT-DADMe-ImmA, EtT-DADMe-ImmA, and p-Cl-PhT-DADMe-ImmA) show similar patterns with small, equivalent thermal enthalpic heat uptakes as all three sites are filled. These data fit well to a single set of identical sites model, supporting the symmetric binding of these inhibitors to trimeric human MTAP (Table 2 and Figure 3C). MT-DADMe-ImmA, EtT-DADMe-ImmA, and p-Cl-PhT-DADMe-ImmA have K_d values of 90, 34, and 10 pM, respectively. The binding of second generation ImmA inhibitors is completely driven by entropy with $-T\Delta S$ values of -15.2 , -16.1 , and -17.6 compared to ΔG values of -13.7 , -14.3 , and -15.0 for MT-DADMe-ImmA, EtT-DADMe-ImmA, and p-Cl-PhT-DADMe-ImmA, respectively (Table 2). Thus, the entropy term dominates binding and the enthalpic contribution is a modest penalty toward the binding energy (ΔG).

The binding thermodynamics of all transition state analogue inhibitors of MTAP establish entropy as the driving force (Figure 4). The enthalpic contribution to binding is unfavorable but is a small fraction of the total binding energy. Increased hydrophobicity of the 5'-group promotes higher affinity to MTAP binding by causing an even more favorable entropy term. The second-generation inhibitors are better mimics of the MTAP transition state and thus exhibit tighter binding than first-generation transition state analogues. However, for all inhibitors tested here, binding is entropy-driven.

Thermodynamics of Adenine Binding. Adenine is a product of MTA phosphorolysis by MTAP and is also of interest since it is found tightly bound on the purified enzyme. ITC data of adenine binding to adenine-free MTAP indicates sequential binding to the three active sites of trimeric MTAP (Table 2 and Figure 3A) to give K_d values of 2.1 and 3 μ M for the first two sites and 80 μ M for the third site. Entropy ($-T\Delta S$) contributes all of the binding free energy (ΔG) for the first two binding sites and accounts for 54% of the binding energy for the third site. Like the transition state analogues, binding of adenine to the initial binding sites is completely driven by entropy although the binding energy provided by the

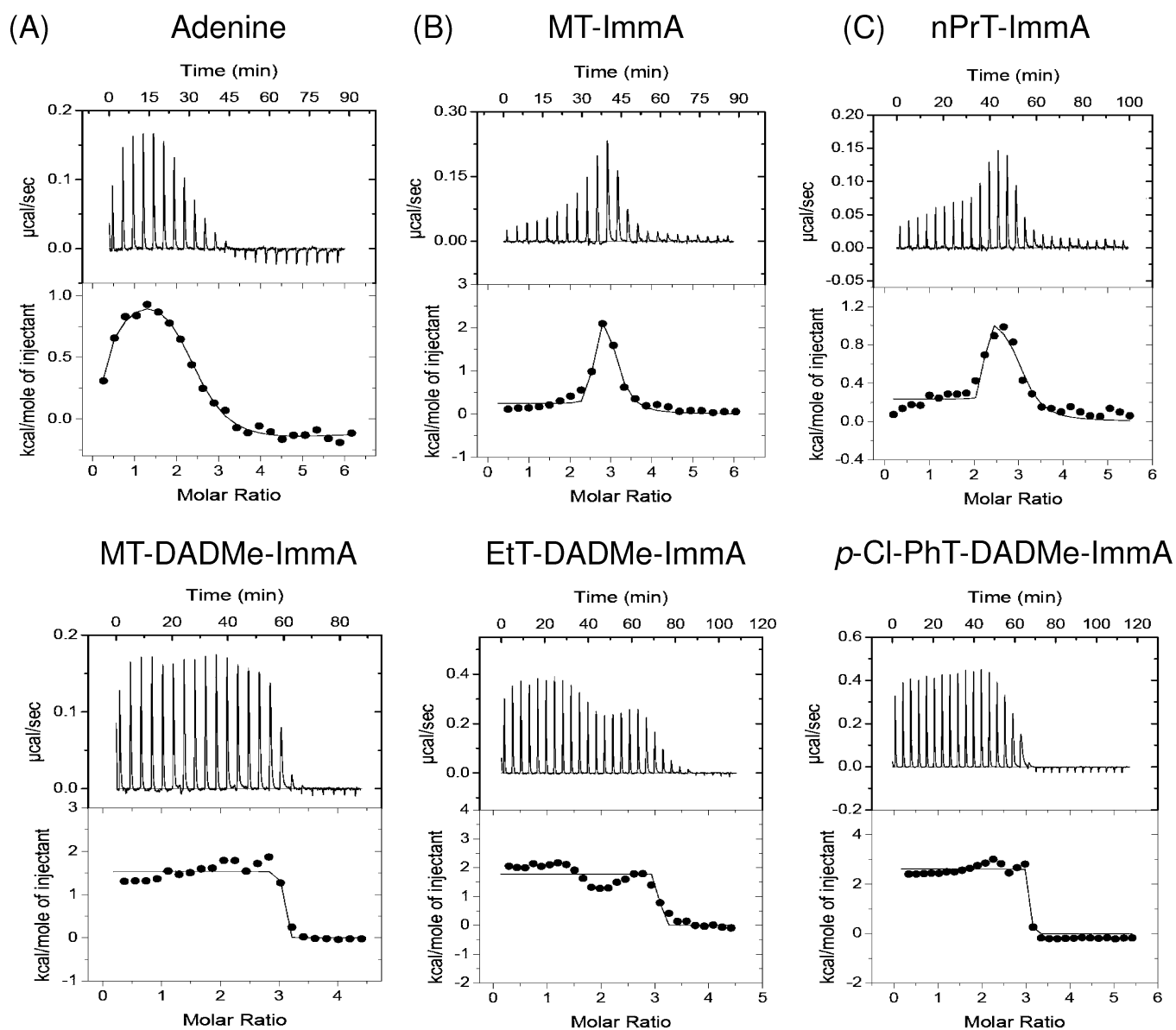


Figure 3. Isothermal titrations of human MTAP with (A) adenine, (B) iminoribitol-based transition state analogues of MTAP, and (C) hydroxypyrrolidine transition state analogues of MTAP (see Figure 1). The bell-shaped curves in panels A, B, and C result from distinct enthalpic contributions upon filling sites 1, 2, and 3.

entropy term (-7.6 kcal/mol for first site) is smaller than found for transition state analogue inhibitors (Figure 4), as expected because of the lack of transition state characteristics for adenine.

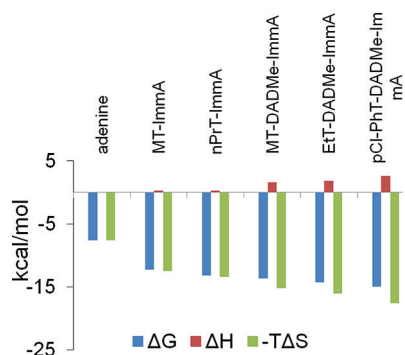


Figure 4. Thermodynamics signatures of inhibitors binding to MTAP.

The ITC values for adenine binding in the micromolar range are inconsistent with tightly bound adenine in purified enzyme but may be explained by tighter binding of adenine at the low temperature and phosphate concentration used for purification.

Heat Capacity of Human MTAP and Inhibitor Binding. Binding enthalpy was measured at temperatures from 15 to 37 °C for MT-ImmA. Inhibitor binding was a linear function of temperature (Figure 5). The heat capacity change (ΔC_p) was determined to be -600 ± 40 cal/(mol K). This large and negative ΔC_p indicates altered hydrophobic effects upon ligand binding and can result from the removal of nonpolar surface from the solvent or the exclusion of bulk water from the enzyme active site.^{24,25} Large and negative ΔC_p values also indicate favorable solvation entropy.²⁶ The ΔC_p of -600 cal/(mol K) indicates the release of water from human MTAP and/or increased system hydrophobic interactions upon inhibitor binding, consistent with the entropy-driven binding observed in the ITC studies.

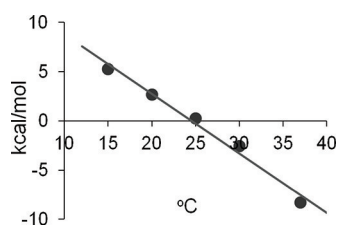


Figure 5. Heat capacity change of MTAP upon binding of MT-ImmA. The slope of the fitted line is $\Delta C_p = -600 \pm 40$ cal/(mol K).

X-ray Crystal Structures of Human MTAP. Crystal structures of apo MTAP:phosphate and the MTAP:*p*-Cl-PhT-DADMe-ImmA:phosphate complex were determined to 1.9 and 2.0 Å resolution, respectively. The asymmetric unit in apo MTAP crystals contains two homotrimers with 3-fold non-crystallographic symmetry, whereas the asymmetric unit of liganded MTAP crystals contains one MTAP subunit positioned about a crystallographic 3-fold axis, which generates a strict 3-fold symmetric homotrimer.

Human MTAP monomer contains 10 β strands and 6 α helices and possesses an α/β-fold consisting of a 7-stranded β-sheet and 6 α-helices. The β-5, β-6, and β-10 from 7-stranded β-sheet extend to create another 6-stranded β sheet (Figure 6A). The active sites of human MTAP are located

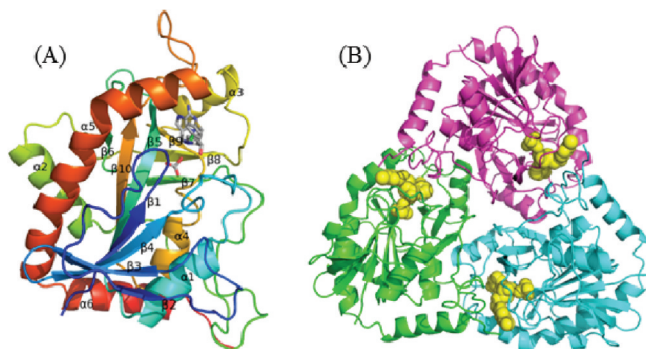


Figure 6. Crystal structure of MTAP. (A) A view of the MTAP monomer. MTAP is color-coded from blue (N-terminus) to red (C-terminus). The *p*-Cl-PhT-DADMe-ImmA and phosphate are included as stick models to show the position of the active site. (B) The monomers of trimeric MTAP are shown in cyan, green, and magenta. The *p*-Cl-PhT-DADMe-ImmA and phosphate are included as space-filling models.

near the interface between two monomers of the trimer, and each trimeric MTAP forms three active sites (Figure 6B). The active site of human MTAP can be divided into three distinct regions corresponding to the purine-, methylthioribose-, and phosphate-binding sites. The purine binding site is located between the backbone of β-5 and β-9 and the side chains of Phe177 and Met196 (Figure 7). Side chains of Ser178, Ile172, Ile194, Asp220, Asp222, and Val231 contribute to the purine binding. The binding site for methylthioribose is formed primarily by hydrophobic residues (Phe177, Met196, Val233, Val236, and Leu237) with contributions from three hydrophilic residues (Thr18, His65, and His 137). Two amino acids in this site (His137 and Val279) are contributed from the adjacent subunit (Figure 7). Phosphate interacts with side chains of Thr18, Thr93, Arg60, His61, and Thr197 and the amide nitrogen groups of Thr18 and Ala94 (Figure 7).

Contacts to *p*-Cl-PhT-DADMe-ImmA. The 9-deazaadenine of *p*-Cl-PhT-DADMe-ImmA is surrounded by the backbone of β-5 and β-9 and the side chains of Ile172, Phe177, Ile194, Met196, and Val231. The side chain of Asp220 forms hydrogen bonds with N6 and N7 of 9-deazaadenine of the inhibitor. Asp222 and Ser178 form hydrogen bonds to the N1 and N6 of 9-deazaadenine via a structural water molecule. The cationic hydroxypyrrolidine of *p*-Cl-PhT-DADMe-ImmA is surrounded by Thr18, His137, Phe177, Met196, Val233, Val236, Leu237, and Val279. The 3'-hydroxyl of the ribocation mimic forms a hydrogen bond with His65 via a structural water molecule. The 1-aza cation of the ribocation mimic forms a 2.8 Å ion pair with the nucleophilic phosphate (Figure 7).

Structural Difference between Apo and Liganded MTAP. The overall crystal structures of apo and liganded MTAP are similar (the C_α rmsd of 0.7 Å for 258 residues in the monomer), except the region from Thr219 to Asn245 and the loop composed of Gly16 to Glu24 (Figure 8A). In the crystal structure of the *p*-Cl-PhT-DADMe-ImmA:MTAP:phosphate complex, the side chains of Val231 (5 Å), Val236 (4 Å), and Leu237 (4 Å) interact with inhibitor through van der Waals interactions. Superposition of apo and liganded MTAP reveals that Val236 and Leu237 of apo MTAP are 6 Å away from the inhibitor and Val231 is disordered. Thus, the binding of *p*-Cl-PhT-DADMe-ImmA induces the bending of the N-terminal end of the Val233 to Asn245 segment of α-5 toward the active site (Figure 8A). In addition, the loop composed of residues 227 to 235, located between α-5 and β-10, is ordered in liganded MTAP but disordered in the apo protein. The ordered loop contacts the side chain of Phe177 and the adjacent subunit. The movement of helix 5 creates the active site cavity (Figure 8B,C). Similar catalytic site features are also observed in crystal structures of MTAP in complex with adenine (PDB ID: 1CB0) or MT-ImmA (PDB ID: 1K27) when compared to the apo protein. The conformational change of the region from Thr219 to Asn245 results in active site closing and facilitates tight binding of the transition state analogue inhibitors. Since transition state analogues are related to productive species encountered along the reaction coordinate, this geometry is likely to be pertinent for catalysis. These conformational changes suggest increased order in the protein, but inhibitor binding is driven by favorable entropy. These changes are associated with ligand binding causing protein order but also inducing the release of water from the active site and from the trimer interface, consistent with entropy-driven ligand binding, despite a more ordered catalytic site region.

Global Conformation of Apo and Inhibitor-Bound MTAP. Analytical sedimentation velocity analysis was used to probe whether inhibitor binding to MTAP would change the global conformation of MTAP. Increased entropy on inhibitor binding with local increased order in the catalytic sites suggests the entropic changes come from water interactions or from global expansion of the trimeric protein volume when complexed with inhibitors. The high precision of the interference scans analyzed by the time-derivative method of analytical ultracentrifugation allows subtle changes in sedimentation rate to be reliably measured. As summarized in Table 3, MT-DADMe-ImmA and *p*-Cl-PhT-DADMe-ImmA binding to MTAP results in faster sedimentation of both complexes even after correction for the increased molecular mass of the complexes. The most direct interpretation of these data is that inhibitor binding globally compacts MTAP. This agrees with

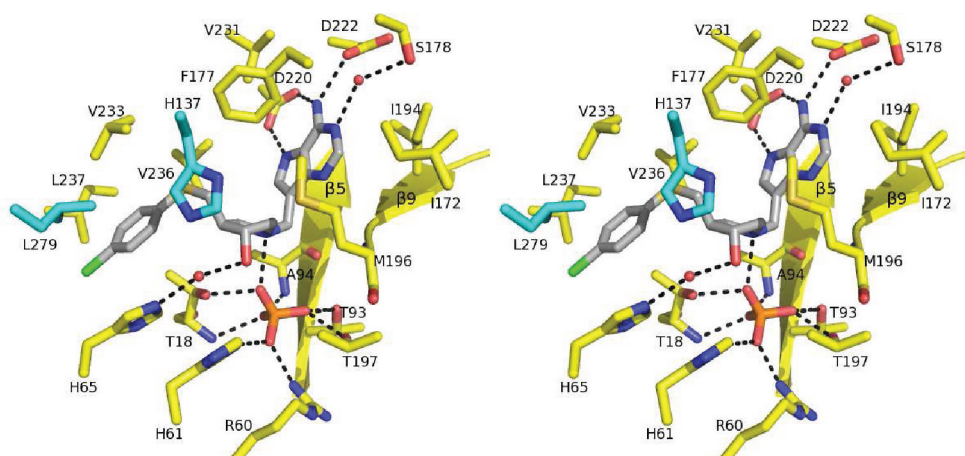


Figure 7. Cross-eyed stereoview of active site of MTAP in complex with *p*-Cl-PhT-DADMe-ImmA and phosphate. The active site residues and the residues contributed from the adjacent subunit are colored in yellow and cyan, respectively. The *p*-Cl-PhT-DADMe-ImmA and phosphate are colored in gray and orange/red, respectively. Hydrogen bonds are indicated as dashed lines.

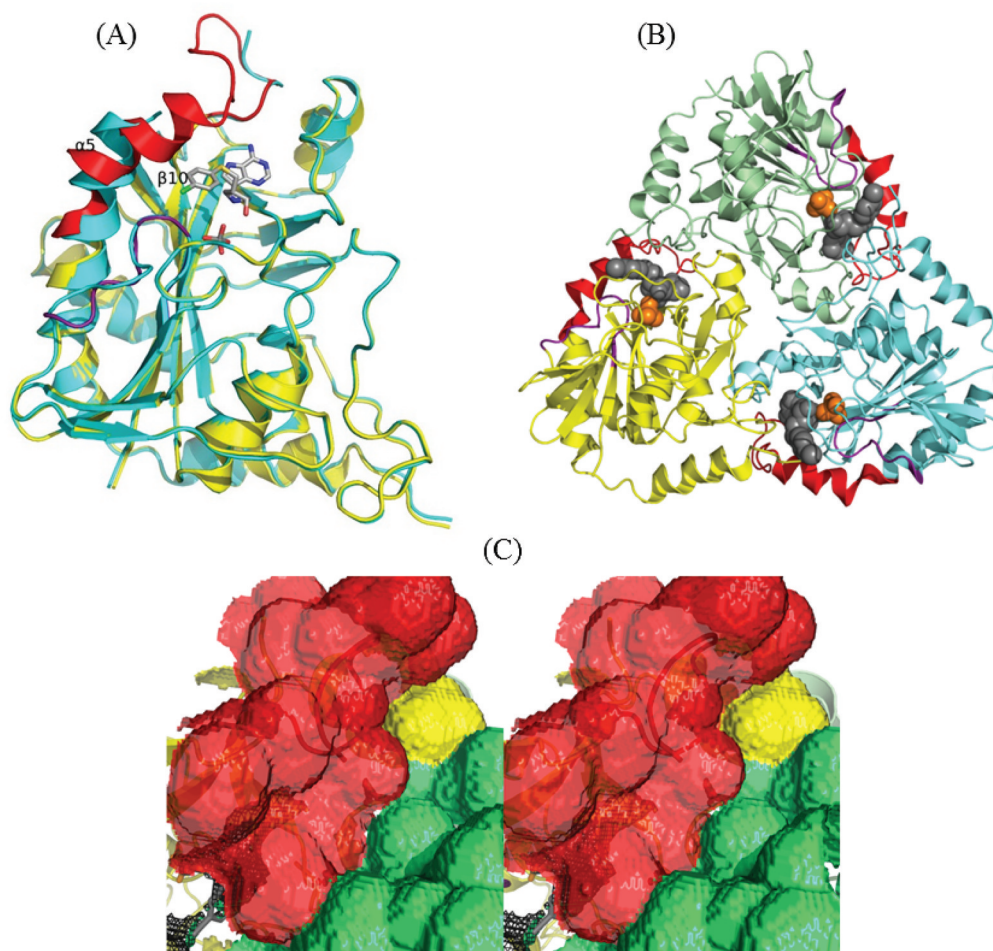


Figure 8. (A) Superimposed structures of apo (cyan) and *p*-Cl-PhT-DADMe-ImmA liganded (yellow) MTAP. The region from Thr219 to Asn245 and the loop of Gly16 to Glu24 of liganded MTAP are highlighted in red and purple, respectively, to emphasize the conformational difference. (B) Monomers of trimeric MTAP in complex with ligand are shown in cyan, green, and yellow. The region from Thr219 to Asn245 and the loop of Gly16 to Glu24 of liganded MTAP are highlighted in red and purple, respectively. The *p*-Cl-PhT-DADMe-ImmA (gray) and phosphate (orange) included as space-filling models. (C) The cross-eyed stereoview of the solvent accessible surface of inhibitor bound MTAP highlighted via a Connolly surface (the molecular surface is generated using 1.4 Å probe). The surface contributed from a protomer, the region from Thr219 to Asn245 and the adjacent subunit are colored in yellow, red, and green, respectively. The enclosed active site pocket is drawn as black mesh.

the increased crystallographic order seen near the catalytic sites with inhibitor bound.

Comparison of Binding Thermodynamics of PNP and MTAP. Human PNP and MTAP belong to the nucleoside

Table 3. Compaction Induced by Transition State Analogue Binding Revealed in the Sedimentation Rate of the MTAP Complexes^a

sample	$S_{(20,w)}$ (normalized)	$S_{(20,w)}$ relative change (%)
MTAP	5.858 ± 0.021	
MTAP + MT-DADMe-ImmA	5.904 ± 0.016	0.79 ± 0.05
MTAP + <i>p</i> -Cl-PhT-DADMe-ImmA	6.012 ± 0.067	2.63 ± 0.12

^aThe sedimentation coefficients determined for replicate experiments were averaged and the relative change in sedimentation coefficients ($S_{(20,w)}$) are reported. The S values of the complexes are normalized for the increased molecular mass of the bound ligands.

phosphorylase-I structural family and are proposed to have evolved from a common ancestor and thus contain homologous structural elements.²⁷ Despite the structural similarity, the catalytic site functions differ in extraordinary ways. For example, the binding thermodynamics of human PNP with an optimized 8.5 pM transition state analogue inhibitor features a ΔH term of -18.6 kcal/mol and a small unfavorable $-T\Delta S$ term.¹² The dominant ΔH term results from the hydrogen-bonding and ion-pair network formed upon inhibitor binding, which closely mimics the interactions at the enzyme transition state. The entropy penalty is readily explained from the increased order parameters of the protein:inhibitor complex.¹² Since the overall structure and transition state analogue inhibitors of MTAP are similar to those of PNP, the thermodynamic properties of transition state analogues binding to MTAP are surprising in being driven completely by entropy with small unfavorable enthalpy terms (Figure 9). The opposite binding thermodynamics for transition state analogues to related enzymes leads to the inescapable conclusion that interactions near the transition states differ substantially for PNP and MTAP despite common protein structural elements.

Comparing the hydrogen bond and ion-pair networks at the active sites of MTAP and PNP with bound transition state analogues reveals similar types and numbers of interactions. However, interactions at the sugar pockets are significantly different near the 5'-positions. In PNP, the primary interaction to the 5'-OH group is a hydrogen bond interaction with His257. In MTAP, the 5'-hydrophobic group is surrounded by Val233, Val236, Leu237, and Val279, forming strong hydrophobic interactions. The removal of those hydrophobic residues from solvent supports a favorable entropy term upon inhibitor binding. In MTAP, His137 is from the adjacent subunit, forming van der Waals contacts near the 5'-position of the sugar with hydrogen bonds to His65. Leu237 is the other residue donated to the catalytic sites of MTAP by the adjacent subunit. In contrast, there are fewer contacts from adjacent subunits in the active sites of PNP. The interactions from neighboring subunit residues in the active site of MTAP are involved in rearrangement of the monomer interfaces upon inhibitor binding. These changes are proposed to contribute to the entropy term by releasing water molecules present in the apoenzyme. Overlays of crystal structures of apo MTAP and its inhibitor complexes do not show significant changes in the positions of Leu237 and His137, but altered dynamics of these regions might also contribute to entropic terms, but only in a local way that still permits compaction of the complex. The most significant conformational change upon inhibitor binding comes from the loop residues 227 to 235 in MTAP. This loop is flexible in the apo protein and allows the bulk solvent to

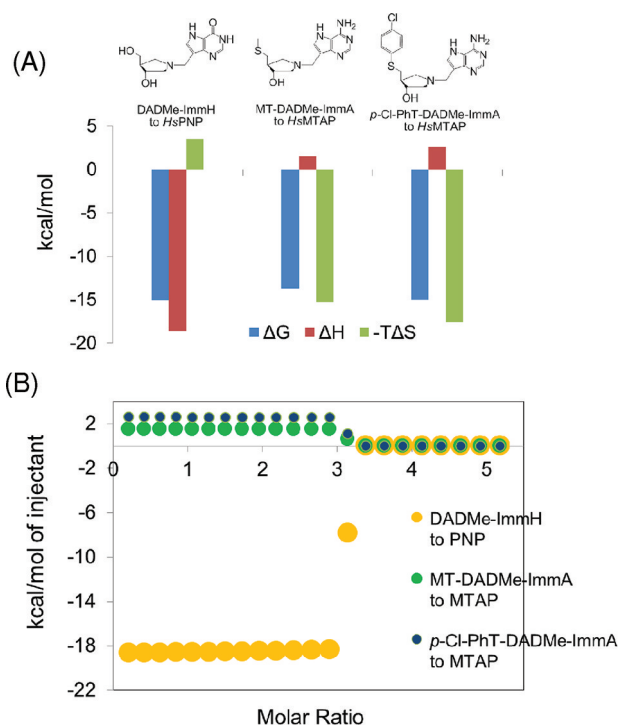


Figure 9. Binding thermodynamics of PNP and MTAP. The thermodynamic signatures of PNP¹² and MTAP are compared for the binding of DADMe-ImmH (K_i of 8.5 pM) and MT-DADMe-ImmA (K_i of 90 pM) in panel A. The binding energy of PNP comes completely from favorable enthalpy with an entropic penalty. The tight binding of MTAP is driven by entropy with a minor enthalpy penalty. Thermodynamic titration data for PNP and MTAP are compared in the simulation in panel B.

reach the active sites. Binding of the inhibitor causes the conformational change of the loop and closes the solvent channel to prevent access of solvent to the active site. The closure of the loop is thus consistent with the entropy-driven binding of the transition state analogue inhibitors.

Structural examination of PNP shows more subtle changes on substrate binding, accompanied by overall shrinkage of the protein as indicated by a 3% change in sedimentation velocity when a tight-binding inhibitor is bound.¹² With MTAP we see a similar change of 2.63% increase in sedimentation velocity with *p*-Cl-PhT-DADMe-ImmA bound. The large hydrophobic group of *p*-Cl-PhT-DADMe-ImmA and loop motion to cause exclusion of water from the catalytic site and from subunit interfaces are dominant entropic forces that overwhelm the obvious enthalpic interactions of the catalytic site, including the formation of a new ion pair between the ribocation mimic and bound phosphate.

By making stable contacts to the enzyme to mimic interactions similar to the transition state, transition state analogues achieve tight binding to their cognate proteins. Here we show that these interactions can be manifested as an entropic change when the changes in water structure overwhelm the enthalpic interactions forming from catalytic site contacts. As two structurally and mechanistically related enzymes show dramatically different effects, it appears that each specific enzyme has novel mechanisms of converting bound complexes toward the transition state.

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Funding

This work was supported by research grants CA135405 and GM41916 from the National Institutes of Health. Financial support was also provided by the Offices of Biological and Environmental Research and of Basic Energy Sciences of the US Department of Energy.

ACKNOWLEDGMENTS

Structural data for this study were measured at beamline X29A of the National Synchrotron Light Source. The authors acknowledge members of the Carbohydrate Chemistry Team of Industrial Research Ltd. (Lower Hutt, New Zealand) for their contribution to the synthesis and supply of transition state analogue inhibitors.

ABBREVIATIONS

MTA, 5'-methylthioadenosine; SAM, S-adenosyl-L-methionine; MTAP, MTA phosphorylase; PNP, purine nucleoside phosphorylase; MTR-1-P, 5-methylthioribose- α -D-1-phosphate; Imma, Immucillin-A; DADMe, 4'-deaza-1'-aza-2'-deoxy-1'-(9-methylene).

ADDITIONAL NOTE

^aThe K_i values listed for the immucillin inhibitors is the equilibrium inhibition constant, previously referred to as K_i^* from slow-onset kinetic studies.

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