Catalytic Site Cooperativity in Dimeric Methylthioadenosine Nucleosidase

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ABSTRACT: S′-Methylthioadenosine/S-adenosylhomocysteine nucleosidases (MTANs) are bacterial enzymes that catalyze hydrolysis of the N-ribosidic bonds of S′-methylthioadenosine (MTA) and S-adenosylhomocysteine (SAH) to form adenine and S-thioribosyl groups. MTANs are involved in AI-1 and AI-2 bacterial quorum sensing and the unusual futolosine-based menaquinone synthetic pathway in Streptomyces, Helicobacter, and Campylobacter species. Crystal structures show MTANs to be homodimers with two catalytic sites near the dimer interface. Here, we explore the cooperative ligand interactions in the homodimer of Staphylococcus aureus MTAN (SaMTAN). Kinetic analysis indicated negative catalytic cooperativity. Titration of SaMTAN with the transition-state analogue MT-DADMe-ImmA gave unequal catalytic site binding, consistent with negative binding cooperativity. Thermodynamics of MT-DADMe-ImmA binding also gave negative cooperativity, where the first site had different enthalpic and entropic properties than the second site. Cysteine reactivity in a single-cysteine catalytic site loop construct of SaMTAN is reactive in native enzyme, less reactive when inhibitor is bound to one subunit, and nonreactive upon saturation with inhibitor. A fusion peptide heterodimer construct with one inactive subunit (E173Q) and one native subunit gave 25% of native SaMTAN activity, similar to native SaMTAN with MT-DADMe-ImmA at one catalytic site. Pre-steady-state kinetics showed fast chemistry at one catalytic site, consistent with slow adenine release before catalysis occurs at the second catalytic site. The results support the two catalytic sites acting sequentially, with negative cooperativity and product release being linked to motion of a catalytic site loop contributed by the neighboring subunit.

Bacterial S′-methylthioadenosine (MTA) and S-adenosylhomocysteine (SAH) are formed in reactions of the polynucleotide pathway and in methyl-transfer reactions where S-adenosylmethionine is the methyl donor. One fate of these products is to undergo N-ribosyl bond hydrolysis from the action of S′-methylthioadenosine/S-adenosylhomocysteine nucleosidases (MTANs). The product adenine can be recycled to the adenine nucleotide pool via adenine phosphoribosyltransferase, whereas S-methylthioribose can be converted to methionine for recycling to S-adenosylmethionine.1 MTA and SAH are product inhibitors of MTAN, production of AI-2 was dramatically reduced in MTAN knockout strains or in the presence of tight-binding inhibitors of MTAN, production of AI-2 was dramatically reduced.1 In other bacterial species, MTAN is essential in an alternative menaquinone synthetic pathway involving futolosine.3−9 Because MTAN is not expressed in humans, it provides a potential target for antibacterial drug design both in QS signaling and in atypical menaquinone synthesis.

In QS, small molecules called autoinducers (AI) are synthesized and released as cell-to-cell signaling molecules.3 AI-1 are acylated homoserine lactones (AHL) that serve as signaling molecules between bacteria of the same species. A byproduct of AI-1 synthesis is MTA, which is also a product of the polynucleotide synthesis pathway. Through product inhibition, MTA is an inhibitor of AHL synthase and polynucleotide synthase. AI-2 is a signaling molecule for QS and is thought to play a role in bacterial communication between different species.1,3 S-Ribosylhomocysteine (SRH) is formed by the action of MTANs on SAH and is the precursor for AI-2 synthesis. Inhibition of MTAN causes MTA and SAH accumulation and is proposed to inhibit both AI-1 and AI-2 production (Figure 1). In MTAN knockout strains or in the presence of tight-binding inhibitors of MTAN, production of AI-2 was dramatically reduced. 12 Recently, MTAN has also been recognized as essential for QS-independent virulent factors in Neisseria meningitides and Staphylococcus aureus.5,6 In other bacterial species, MTAN is essential in an alternative menaquinone synthetic pathway involving futolosine.7−9

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result is interpreted as the three sites acting sequentially, and interruption of the catalytic site cycling by a tight-binding inhibitor at one site causes full inhibition. However, in the crystal structure, all three sites can be saturated with inhibitor to give human PNP as a symmetrical trimer with the three active sites located at the interfaces of adjacent subunits. Another member of this family is human 5′-methylthioadenosine phosphorylase (MTAP; 5′-methylthioadenosine + phosphate → adenine +5-methylthio-α-D-ribose 1-phosphate). This trimeric structure is similar to PNP, but the three catalytic sites are independent such that filling one of the catalytic sites with a tight-binding inhibitor leaves the other two fully active. Thus, filling one or two of the three sites with inhibitor causes 1/3 or 2/3 inhibition of full catalytic activity.

In the structurally related trimeric PNP and MTAN enzymes, the patterns of catalytic site cooperativity differ substantially. A similar analysis has not been reported for the related MTAN dimeric enzyme family. Here, we establish cooperativity and investigate the possibility of oligomerization changes by covalent coupling of the subunits. We find no evidence for subunit dissociation or aggregation. A functional dimer is consistent with the crystal structures of MTANs. Subunit interfaces reveal substantial interactions in the apoenzyme and increased interactions when catalytic site ligands are bound. Here, we characterize the interactions of SaMTAN dimer catalytic site interactions using kinetics, chemical reactivity, and transition-state analogue inhibitors as probes.

Approximately 30 crystal structures of MTANs from several bacterial and plant species are available in the PDB. All are homodimers with the active sites near the dimer interfaces. In the closed complexes with the catalytic sites filled, the catalytic site cavities are covered by a loop from the adjacent monomer. The center of these loops contains a phenylalanine that is located over the filled active site on the adjacent subunit (Phe104 in SaMTAN). The loop has been proposed to isolate the catalytic site from bulk solvent, and this loop also interacts with the 5′-methylthio group or homocysteine group of reactants. Finally, it serves as a potential link for cooperative communication between subunits.

On the basis of kinetic isotope effects and computational chemistry, MTAN transition-state structures are ribocationic at C1′ with a neutral, N7-protonated adenine as the leaving group. MTANs also stabilize a specific water molecule adjacent to C1′ as the incipient nucleophilic group. The chemical reactivity of ribocations is extremely high, requiring critical positioning of C1′ and the water molecule to prevent reaction of the ribocation with nearby protein nucleophiles. The loop is likely to play a role in stabilizing the reactants in a geometric position that permits them to reach the transition state with the appropriate water–ribocation geometry.

Figure 1. MTAN in quorum sensing (top) and menaquinone synthesis (bottom).
Isothermal titration calorimetry (ITC) studies suggested that dimeric MTANs from *Escherichia coli*, *Salmonella enterica*, and *Vibrio cholerae* all demonstrate negative cooperativity for inhibitor binding at the first and second sites. However, the role of catalytic site cooperativity was not extended to the catalytic function with normal substrates. In this study, we show that the monomers of *S. aureus* MTAN (SaMTAN) have negative cooperativity upon catalytic site filling and this cooperativity extends to the catalytic function of SaMTAN.

**MATERIALS AND METHODS**

**Chemicals.** MT-DADMe-ImmA was synthesized as previously described and generously provided by Dr. Gary B. Evans of the Carbohydrate Chemistry Team, Ferrier Institute, Victoria University of Wellington, Lower Hutt, New Zealand. Xanthine oxidase (Grade III) and 5′-deoxy-5′-methylthioadenosine (MTA) were purchased from Sigma-Aldrich (Saint Louis, MO). 5,5′-Dithiobis-2-nitrobenzoic acid (DTNB) was purchased from Sigma-Aldrich (Milwaukee, WI). All other chemicals were purchased at the highest purity commercially available and used without further purification.

**Enzyme Purification.** Native and mutant SaMTANs were purified as described earlier. In brief, a plasmid containing SaMTAN and encoding an N-terminal His6 tag was transformed into BL21 (DE3) *E. coli* cells. Bacterial cells were grown to an OD of 0.6 at 600 nm before induction with 1 mM IPTG. Cells were harvested by centrifugation after 20 h of additional growth at 20 °C. Cells from 6 L of culture were broken by sonication, and the soluble portion was harvested after centrifugation. The sample was applied to a 20 mL Ni-NTA column that was pre-equilibrated with 50 mM HEPES (pH 7.6). The column was washed with 200 mL of buffer containing 50 mM HEPES (pH 7.6) and 60 mM imidazole. SaMTAN was eluted from the Ni-NTA column by gravity flow with 50 mL of buffer containing 50 mM HEPES (pH 7.6) and 250 mM imidazole. The protein was concentrated to ~10 mL and dialyzed against 100 mL of buffer containing 50 mM HEPES (pH 7.2), 100 mM NaCl, and 1% (w/v) charcoal with three changes. The protein co-purifies with tightly bound adenine, and the charcoal step is required to remove this adenine. The protein was further purified using a Superdex 200 (26/60) gel-filtration column. The purified SaMTAN reaches >95% purity by this method, and the protein has no bound adenine. The concentration of SaMTAN was determined by adsorption at 280 nm, and the extinction coefficient was determined by the ProtParam program (corrected using the Pace method).

**Kinetic Assays and Inhibitor Titration.** Kinetic assays for inhibitor interactions were performed at 100 μM MTA in 50 mM HEPES, pH 7.2, and the formation of adenine was monitored by the absorption decrease at 274 nm. The MTAN catalytic activity was also detected in continuous assays at 25 °C by monitoring the conversion of adenine to ATP and detection by firefly luciferase (PerkinElmer) in 50 μL reaction mixtures using an assay based on published methods. Differences included addition of PPDK (pyruvate phosphate dikinase) and APRTase (adenine phosphoribosyltransferase) to 780 and 310 μM per well, respectively. MTAN was added to a final concentration of 0.02 to 0.3 nM. MTA concentrations ranged from 0.013 to 4.0 μM. Direct comparisons of catalytic rates from the 274 nm direct kinetic assay and the luciferase assay gave relative *kcat* values of 10.2 and 1.8, respectively, because of the buffer and coupling reagents used in the luciferase assay. All kinetic results are normalized to the rates for the direct kinetic assay.

The concentration of MT-DADMe-ImmA was determined by the absorption at 275 nm with an extinction coefficient of 8500 M⁻¹ cm⁻¹. SaMTAN was incubated with MT-DADMe-ImmA for 30 min with an enzyme concentration of 50 μM prior to activity assay. Assays were initiated by adding 3.5 μL of the enzyme—inhibitor solution to 1 mL assay solutions. The rate progression curves were linear, indicating no inhibitor loss from the SaMTAN complex during the assay. Data were fitted using KaleidaGraph.

**Isothermal Titration Calorimetry Studies.** The binding of MT-DADMe-ImmA (600 μM) to SaMTAN (40 μM) was measured with a VP-ITC microcalorimeter (MicroCal) at 25 °C. The inhibitor and enzyme were in the same buffer containing 30 mM sodium phosphate (pH 7.2) and 100 mM NaCl. Other experimental details were as described previously. Titration of MT-DADMe-ImmA into enzyme-free buffer was measured and used as the control. No significant heat signal was observed. Data were fitted to the two sets of sites model according to the user's manual for the VP-ITC microcalorimeter, where a K1 value of 1.4 nM was fixed for the first site.

**Mutagenesis and Cloning.** Covalently linked heterodimer or covalently linked native dimers of SaMTAN were generated by PCR amplification of the N-terminal encoding region using primer pair 1 (forward: TTTCTAGAAGGAGTAA-AACATATG and reverse: CACCTTCTGGATCCACCT-CCTCGGAACCTCAGGCGCAGCAAATT-GGCTAACAGAGCCTCG) and PCR amplification of the C-terminal encoding region using primer pair 2 (forward: TTCCGGAGGAGTGGATCGAAGGTGGAACCTC-GGAGCTACATGATCGGCGATTTGCTAT and reverse: GGCGAGGTATCACAATTTGCG). The linear DNAs from both PCR reactions were mixed and joined by ligation. The resulting DNA was digested using XbaI and XhoI and incorporated into expression vector pJ214 from DNA2.0.

**DTNB Labeling.** MT-DADMe-ImmA and F104C/C181S SaMTAN were incubated for 30 min at stoichiometries of 0.0, 0.2, 0.5, 0.8, and 1.5 per enzyme subunit (catalytic site), respectively. Reaction mixtures were equilibrated at 25 °C, and reactions were initiated by adding 2 mM DTNB in 50 mM HEPES, pH 7.2, to cuvettes to a final concentration of 300 μM DTNB and 23 μM F104C/C181S SaMTAN. Reactions were monitored continuously in a CARY 300 spectrophotometer with cell changers to follow the absorbance increase at 412 nm.

**Rapid Chemical-Quench Experiments.** Rapid chemical-quench experiments were carried out using an apparatus from KinTek (Model RQF-3) whose temperature was maintained by circulating ice water (0 °C). The reaction was initiated by rapid mixing of SaMTAN (0.1 mM) and MTA (1 mM). After appropriate delay times, the SaMTAN reaction was quenched using 5 M HCl from a third syringe, and product (adenine) formation was quantitated by UPLC calibrated to a standard curve. Using KaleidaGraph (Synergy Software), the rapid-burst phase for formation of adenine was fitted to single exponential (*kburst*), and the linear portion of the curve was fitted to a straight line (*klinear*), yielding values of 52 ± 18 and 1.2 ± 0.2 s⁻¹, respectively.

**Solvent Viscosity Studies.** Solvent viscosity effects were performed using glycerol or sucrose as the viscosogens. To achieve relative viscosity values of 1, 2, 3 and 4, the concentrations of glycerol were 0, 24, 35, and 42% (w/w), and for sucrose, the concentrations were 0, 21, 29, and 34% (w/w).
RESULTS AND DISCUSSION

Steady-State Kinetic Analysis of SaMTAN. Kinetic analysis of SaMTAN saturation with MTA was unusual in giving a negative cooperativity (Hill plot = 0.9, not shown) and high apparent affinity (nanomolar) for substrate saturation (Figure 2).

The high affinity for MTA required use of a sensitive luciferase assay for adenine quantitation.\textsuperscript{21,22} Substrate saturation curves did not conform to the Michaelis–Menten equation ($v = \frac{k_{cat}A}{(K_m + A)}$) or to the equation for two catalytic sites acting independently with distinct kinetic constants ($v = \frac{k_{cat1}A}{(K_{m1} + A)} + \frac{k_{cat2}A}{(K_{m2} + A)}$). The data conformed to a kinetic mechanism where the first site to fill is active but when both sites are filled, distinct kinetic parameters are observed according to the expression first named by W. W. Cleland as a “2/1” inhibitor function\textsuperscript{23} and later adapted to cooperative multiple catalytic sites in the form $v = \frac{k_{cat}A}{(K_m + A)} / \left(\frac{A + k_{cat2}K_{m2}}{k_{cat2}}\right) / \left(\frac{A + k_{cat1}K_{m1}}{k_{cat1}}\right)$. This equation, $v$ is the initial reaction rate, $A$ is substrate concentration, $k_{cat}$ is the rate of product formation from saturated enzyme (EA\textsubscript{2}), $k_{cat1}$ is the rate of product formation from the enzyme with one site filled (EA\textsubscript{1}), $K_m$ is the Michaelis constant for the filling the second substrate site, and $K_m$ the Michaelis constant for filling the first binding site. Least-squares fits of the data to the 2/1 equation gave $k_{cat2}/k_{cat1} = 4.3, K_{m1} = 0.1\, \mu$M, and $K_{m2} = 0.9\, \mu$M. The rate of product formation from the EA\textsubscript{2} complex ($k_{cat2}$) is 10.2 s\textsuperscript{-1}, and from the EA\textsubscript{1} complex ($k_{cat1}$), is 2.4 s\textsuperscript{-1} (1.8 and 0.42 s\textsuperscript{-1} in the luciferase assay, Figure 2, see Materials and Methods). We sought to validate the steady-state kinetic analysis experimentally by direct physical chemistry measurements, including residual catalytic site activity as one site is filled with a transition-state analogue.

Titration of SaMTAN with Tight-Binding Inhibitor. Purified apo-SaMTAN was titrated with MT-DADMe-ImmA, a transition-state analogue with a dissociation constant of 1.4 nM (Figure 3).\textsuperscript{2} MT-DADMe-ImmA is a close analogue of the transition state for hydrolysis of MTA by MTANs with a S'-methylthioribocatic center and a N7-protonated adenine leaving group. The enzyme (at a concentration of 50 μM) was incubated with the inhibitor for >10 min, and samples were taken to initiate steady-state assays. The assay mixtures contained relatively high enzyme concentrations (100–500 nM) to prevent fractional dissociation of the inhibitor during assays. With a $K_i$ value of 1.4 nM, less than 1% of the MT-DADMe-ImmA is expected to dissociate from SaMTAN during the 0.5 to 2 min assay time. This assumption was supported by linear initial reaction rates in a direct recording spectroscopic assay. MTAN catalytic activity decreased as a linear function of inhibitor concentration until saturation of the first catalytic site was achieved. With one site filled, 80% of the total catalytic activity was lost (Figure 3). Titration of the second site led to complete inhibition of catalytic activity at a stoichiometry of 1:1 of inhibitor to catalytic sites. Although both subunits have high affinity for the inhibitor relative to enzyme and inhibitor concentrations in the titration, binding to the second site has lower affinity than to the first. In the crystal structure, SaMTAN is a symmetric dimer. Thus, the decreased inhibitor affinity of the second site as the first catalytic site is filled can be attributed to negative cooperativity and can be correlated with catalytic site activity. When one site only is filled with substrate (EA\textsubscript{1}), catalysis is $\frac{1}{4}$ of the rate from EA\textsubscript{2}. When one site is filled with inhibitor, the IEA\textsubscript{1} complex gives rise to product at $\frac{1}{4}$ of the rate from EA\textsubscript{2}. Another test of negative cooperativity is direct analysis of the thermodynamic parameters for catalytic sites in ITC titration experiments.

Site-Specific Thermodynamics of Tight-Binding Inhibitor. Thermodynamic features of the two catalytic sites were resolved by isothermal titration calorimetry (ITC) experiments for SaMTAN binding to MT-DADMe-ImmA at 25 °C (Figure 4). ITC technology has a lower limit of ~10 nM for $K_d$ values to permit full thermodynamic analysis by titration.\textsuperscript{26} The kinetically determined $K_d$ value is 1.4 nM for MT-DADMe-ImmA binding; therefore, active-site titration is near-stoichiometric with little accumulation of free ligand. The ITC data used the kinetically determined $K_d$ value (1.4 nM) as a fixed constant into the fitting algorithm for binding to the first site of the SaMTAN dimer. The $K_d$ value of the second site was a variable and was fitted from the titration data to give a value of 9 nM. Thus, affinity of MT-DADMe-ImmA to the second subunit decreased 6-fold relative to the first site. Binding of MT-DADMe-ImmA to the first site yielded a $\Delta H$ of $-10.1$ kcal/mol and a $-T\Delta S$ of $-1.9$ kcal/mol.

Figure 2. Catalytic activity of SaMTAN dimer as a function of MTA using the coupled assay for detection of adenine. The line is the best fit to the experimental points for the 2/1 equation described in the text. There are 21 experimental rate measurements in the fitted curve, partially obscured by overlap. The wide substrate range is needed to define the saturation of both catalytic sites. The kinetic constants from the fit are indicated in the inset.

Figure 3. Residual catalytic activity ($v/v_0$) as a function of MT-DADMe-ImmA titration of SaMTAN. Symbols $v$ and $v_0$ are reaction rates in the presence and absence of inhibitor, respectively. Approximately 80% inhibition occurs when the first site is filled, and complete inhibition occurs as the second site is filled. The abscissa is the ratio of MT-DADMe-ImmA to SaMTAN dimer. The line is a linear fit to the experimental points as the MT-DADMe-ImmA/SaMTAN ratio increases to 1.0.
Hydrophobic rearrangements around the unbound second namic di−entropy are attributed to dynamic components, water exclusion, interactions from the ligand binding, and the contribution of 3.8 kcal/mol than the second site. Enthalpic contributions are Sa0.001 min with DTNB to give a single exponential rate constant of 0.095 substrate interaction is similar to the Δaabscissa is the ratio of MT-DADMe-ImmA to ff for MT-DADMe-ImmA binding. With these different, it is significant that the ratio of MT-DADMe-ImmA to MT-DADMe-ImmA is 10.2 s−1 and a dissociation constant of 1.4 nM, the off-rate is 0.014 s−1, 40 times faster than the C104 chemical reactivity rate of 0.021 min−1. Thus, the inhibitor equilibrates between sites on the time scale of a few minutes, whereas the DTNB reaction occurs on the time scale of 2 hours for half-saturated enzyme (Figure 5).

Pre-Steady-State Burst Kinetics for SaMTAN. The kcat steady-state rate of MTA hydrolysis by SaMTAN is 10.2 s−1, and we tested if this rate is limited by chemistry and/or site–site interactions. We hypothesized that adenine product release might limit steady-state kinetic rates because the enzyme is isolated with tightly bound adenine (see below). Likewise, the negative cooperativity between sites questions if both sites can react at the same rate in the presteady state. Rapid mixing of SaMTAN with near-saturating MTA followed by chemical quench at 25 °C gave conversion of one catalytic site equivalent of MTA to adenine (half the sites reactivity) within the 3 ms time resolution of the experiment. At 0 °C, it was possible to measure the rapid formation of adenine at one of the two catalytic sites with an observed rate of 52 ± 18 s−1 followed by a steady-state rate of product formation of 1.2 ± 0.2 s−1 (Figure 6). The catalytic burst for the single-site on-enzyme chemistry is 43 times faster than the steady-state rate at 0 °C. Assuming a similar temperature coefficient for steady-state and pre-steady-state rates for extrapolation to 25 °C, the pre-steady-state burst is estimated to be 442 s−1 followed by the steady state rate of 10.2 s−1. The slow steady-state rate is consistent with slow release of product from the first catalytic site being required for catalytic function of the second site. SaMTAN binds adenine tightly and copurifies with 0.4–0.6 mol tightly bound adenine per enzyme dimer. This is removed before experiments (see Materials and Methods). We
propose that the slow release of adenine from the first catalytic site is the rate-limiting step in steady-state catalysis. Because only one site reacts in the rapid-burst phase, the result demonstrates sequential catalytic site function. Binding at both sites of MTANs is known to occur from the steady-state kinetic model and the two-site binding with MT-DADMe-ImmA and SaMTAN dimer complexes (Figures 2 and 3) and substrate analogues.15 However, only one site is active at a time, and product release from the first site (C104 loop opening), or something with the same rate constant, is required to permit catalysis at the second site. The steady-state cycle suggests that adenine release gives rise to the observed 10.2 s⁻¹ steady-state rate with the sites acting sequentially. If product release is rate

Table 1. Kinetic Constants of Native and Engineered Constructs of SaMTAN

| SaMTAN construct | \( k_{\text{cat}} \) (s⁻¹) | \( K_m \) (μM) | \( k_{\text{cat}}/K_m \) (M⁻¹ s⁻¹) |
|------------------|----------------|---------|-----------------|
| native homodimer | 10.2 ± 0.1 | 0.9 ± 0.3 | 1.13 × 10⁷ |
| F104C/C186S homodimer | 7.2 ± 0.4 | 2.9 ± 1.2 | 2.48 × 10⁶ |
| native fusion homodimer | 8.3 ± 0.2 | 1.3 ± 0.1 | 6.38 × 10⁵ |
| E173Q + native fusion heterodimer | 2.0 ± 0.2 | 0.5 ± 0.2 | 4.0 × 10⁶ |
| E173Q homodimer | <0.1 | not measured | not measured |

Figure 5. DTNB labeling of C181S/F104C SaMTAN. Top: the active-site entrance is covered by F104, which was replaced with a cysteine for DTNB-labeling study. Bottom: Reaction of C181S/F104C SaMTAN complexes with DTNB. The reaction rates are for the reaction of DTNB with MT-DADMe-ImmA and SaMTAN dimer complexes at ratios of no inhibitor (magenta), 0.4:2.0 (green), 1.0:2.0 (yellow), 1.6:2.0 (blue), and 2.0:2.0 (black). All traces are corrected for the background rate of hydrolysis of DTNB.

Table 1. Kinetic Constants of Native and Engineered Constructs of SaMTAN
limiting, then the catalytic rate would be expected to be sensitive to solvent viscosity.

Viscosity Effects Support Adenine Product Release as the Slow Step. In enzyme systems where rate is governed by diffusion, including product release, microviscogens like glycerol or sucrose are expected to alter the diffusion rate and change the kinetic rate as a linear function of viscosity. The results support adenine release as a diffusion-controlled rate-limiting step during steady-state catalysis. Addition of glycerol to increase the viscosity of assay solutions slowed the $k_{\text{cat}}$ for SaMTAN as a linear function of viscosity in glycerol ($\eta/\eta_0$) (Figure 6). A similar experiment used sucrose as a second microviscogen and gave similar results (not shown).

Chimeric Linked SaMTAN with One Functional Catalytic Site. Pre-steady-state kinetics of MT-DADMe-ImmA inhibitor binding at one site and C104 chemical reactivity revealed aspects of negative cooperativity between subunits. We explored the properties of a SaMTAN in which one subunit is rendered catalytically inactive, the second is active, and the subunits are covalently linked. This approach explores one-site reactivity while preventing subunit-dissociation effects (Figure 7).

The inactive monomer contained an E173Q mutation, the dimer of which reduced $k_{\text{cat}}$ by more than 2 orders of magnitude (Table 1). To ensure active/inactive subunit stoichiometry and to decrease the possibility of subunit dissociation, the subunits were covalently linked by a 20 amino acid linker rich in serine and glycine to maximize linker flexibility (sequence: ASGAGGSEGEGGTSGAT, named L20). The construct was expressed as a fusion protein with the E173Q inactive mutant in the N-terminal peptide and the native subunit in the C-terminal peptide. In the crystal structure of native MTANs, the distance between the N-terminus of one subunit to the C-terminus of the second subunit is 50 Å, and the fully extended length of the linker L20 is 70 Å. As a control, native MTAN monomers linked by L20 were also expressed and characterized. Its catalytic activity was similar to native MTAN (Table 1). Compared with native cross-linked SaMTAN, heterocross-linked-SaMTAN had a comparable $K_m$ value, but the value of $k_{\text{cat}}$ was 24% of the control enzyme. During catalysis, only the native subunit of the hetero-cross-linked-SaMTAN is active. It lacks the opportunity to alternate catalytic site action because of the inactive E173Q site. Chimeric SaMTAN does not benefit from the cooperative catalytic interaction of the neighboring subunit. Fused-dimer native SaMTAN has a $k_{\text{cat}}$ of 8.3 s$^{-1}$, whereas the hetero-cross-linked-SaMTAN has a $k_{\text{cat}}$ of 2.0 s$^{-1}$ for its functional catalytic site. This is the same value determined for $k_{\text{cat1}}$ in the kinetic analysis (Figure 2). Because of limited expression and stability of this construct, we were unable to complete ITC and pre-steady-state kinetics with this construct.

Correlation of Structures with Function. Sui et al. reported the crystal structure of SaMTAN and compared it to E. coli MTAN, concluding that the proteins exhibited identical modes of inhibitor binding. A recent structure of S. enterica MTAN, which shares 53% sequence identity with SaMTAN,
shows dramatic structural changes of several regions upon the
binding of either substrate or the MT-DADMe-ImmA
inhibitor.27 The crystal structure geometry of empty catalytic
sites (apoenzyme) in EcMTAN and SeMTAN is open and similar
if empty or if adenine-only is bound.15,16 Thus, the resting
enzyme with one adenine bound is in a similar open catalytic
site geometry to apoenzyme, and the empty second subunit is
prepared to bind substrate, to catalyze the reaction, to facilitate
adenine departure from the first substrate, and to proceed with
substrate binding and chemistry at the first subunit while the
second subunit is cleared by the motion of the 104 loop.

In the earlier structure–function analysis of SaMTAN, Sui et
al.16 reported a $k_{cat}$ of 0.00973 s$^{-1}$ for the enzyme using MTA as
substrate in an assay oxidizing adenine with xanthine oxidase and
coupling the reaction to the reduction of 2-(4-iodophenyl)-3-(4-
nitrophenyl)-5-phenyltetrazolium chloride to form formazan
with detection at 470 nm. As this chemical rate is 1048-fold
slower than we measured for the direct observation of adenine
formation by SaMTAN, this widely used formazan assay is
perhaps not ideal for kinetic investigation of MTANs. The major
findings from the crystal structure of SaMTAN are not affected
by this difference,16 but the catalytic differences need to be
considered in evaluating catalytic efficiency.

Sequential Mechanism for SaMTAN. Kinetic, binding,
thermodynamic, and mutational analysis indicate that both
subunits of SaMTAN can function alone or when its neighbor is
filled. When both are filled, catalysis occurs sequentially,
facilitated by slow product release, presumably by the rate-
limiting 104 loop motion to open the catalytic site for adenine
release. When only one catalytic site of SaMTAN is filled with
substrate, the rate is slower. The high affinity of the first site (0.1
$\mu$M) permits the enzyme to scrub the organism of MTA and
SAH but at a reduced turnover rate. At higher substrate
concentrations (above 1 $\mu$M), where the second site is also filled,
the enzyme can remove substrates at a substantially higher rate
(Figure 8). For the first catalytic turnover with saturated enzyme,
only one site reacts at 442 s$^{-1}$, and catalysis does not occur at the
second site until product release occurs from the first site, a slow
10.2 s$^{-1}$ process. Thus, product release at the first site governs
chemistry at the second site.

■ CONCLUSIONS

Structural similarity exists at the level of tertiary protein folds
between the dimeric MTANs and the mechanistically related
purine nucleoside phosphorylases (PNPs) and methylthioadeno-
sine phosphorylases (MTAPs).10,11 Human PNP and MTAP
are trimers with distinct subunit communication. PNP steady-
state kinetics are Michaelis–Menten; however, the complete
inhibition of the trimer with one site filled with tight-binding
inhibitor establishes an obligatory sequential catalytic site
function.12 Human MTAP is a closely related structural trimer
of the human PNP but has catalytic sites acting independently.14
SaMTAN follows neither of the catalytic site interaction patterns
of its structural relatives. Instead, dimeric SaMTAN has
cooperative sites such that filling one site gives rise to physical
and kinetic properties that are different at the catalytic sites when
both catalytic sites are filled. SaMTAN shows rapid on-enzyme
chemistry at 442 s$^{-1}$ when exposed to saturating substrate. The
rapid reaction is unusual in that it occurs at only one of the two
catalytic sites. The second site forms product at 10.2 s$^{-1}$,
proposed to be the rate of product release. Thus, chemistry
occurs at the saturated second site only as product is released

Figure 8. Catalytic site chemistry and cooperativity for SaMTAN. Monomers of the dimer are shown in gray and yellow, where S represents the MTA
substrate, P represents the products, and In represents the transition-state analogue. In the top reaction sequence, the burst kinetic rates at 25 °C are
shown. The enzyme dimers are symmetric; thus, the first ligand to bind can bind to either catalytic site, and in substrate-saturated enzyme, either site can
react first, leading to branched pathways. For simplicity of illustration, these statistical paths are not shown. Product release of one subunit is necessary
before chemistry can occur at the second subunit.
from the first. This necessarily results in a sequential reaction sequence that alternates between the two sites.

Tight substrate binding and slow product release for dimeric SaMTAN results in an enzyme of high catalytic efficiency with catalytic site cooperativity both for substrate binding and catalytic efficiency. These catalytic site differences are structural, as evidenced by distinct entropic and enthalpic components and similar properties in chimeric enzyme and enzyme with one site blocked by tight-binding inhibitors. The kinetic cooperativity described here for SaMTAN may extend to other MTANs, as ultrasensitive kinetic approaches are required to quantitate these catalytic site properties.

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**Notes**

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