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Capturing Unknown Substrates via *in Situ* Formation of Tightly Bound Bisubstrate Adducts: *S*-Adenosyl-vinthionine as a Functional Probe for AdoMet-Dependent Methyltransferases

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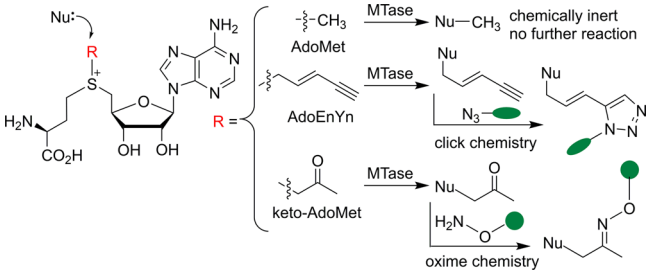
Supporting Information

ABSTRACT: Identifying an enzyme's substrates is essential to understand its function, yet it remains challenging. A fundamental impediment is the transient interactions between an enzyme and its substrates. In contrast, tight binding is often observed for multisubstrate-adduct inhibitors due to synergistic interactions. Extending this venerable concept to enzyme-catalyzed *in situ* adduct formation, unknown substrates were affinity-captured by an *S*-adenosyl-methionine (AdoMet, SAM)-dependent methyltransferase (MTase). Specifically, the electrophilic methyl sulfonium (alkyl donor) in AdoMet is replaced with a vinyl sulfonium (Michael acceptor) in *S*-adenosyl-vinthionine (AdoVin). Via an addition reaction, AdoVin and the nucleophilic substrate form a covalent bisubstrate-adduct tightly complexed with thiopurine MTase (2.1.1.67). As such, an unknown substrate was readily identified from crude cell lysates. Moreover, this approach is applicable to other systems, even if the enzyme is unknown.

Often dubbed as nature's machineries, enzymes play essential roles in biology and diseases. Naturally, it is critical to know what each machine breaks and builds; in other words, the substrates and products of each enzyme. Yet identifying the substrates of each enzyme remains challenging, notwithstanding rapid advancements in genomics, proteomics, and structural biology.[1] One common approach is to facilitate the detection of the products of an enzyme. Toward this end, substrate surrogates containing traceable tags are widely employed, exemplified by *S*-adenosyl-methionine (AdoMet or SAM)-dependent methyltransferases (MTases).[1f,2] As shown in Scheme 1, the methyl group in AdoMet has been replaced by alkyne, ketone, and other functional groups, which label nucleophilic substrates via the transfer of these traceable alkyl groups; subsequently, the resulting products can be labeled via click (azide-alkyne) or oxime (ketone-hydroxylamine) chemistry.[1]

Scheme 1. Methyltransferase-Catalyzed Transfer of a Methyl (AdoMet, Natural Substrate), Alkyne, or Ketone Group (Substrate Surrogates)[a]



[a]The traceable products can be detected via click or oxime chemistry.

In practice, a major limitation for such an approach is that substrates of multiple enzymes ("Nu" in Scheme 1) in the same family may be labeled nonspecifically, thereby the direct association between a particular substrate-enzyme pair cannot be readily established. This is exacerbated for the large family of methyltransferases that catalyze over 300 different reactions with considerable overlapping of substrates. For instance, more than 50 protein lysine and 9 arginine methyltransferases exist in humans alone.[1b−d,f,g]

Conceptually, the interaction between an enzyme and its substrates or products is transient, i.e., being a catalyst, an enzyme does not form a long lasting complex with either its substrates or products, as illustrated in Scheme 2a. As a result, even traceable products often cannot be directly linked to a particular methyltransferase.

A direct, and conceptually distinct, approach is to capture the nucleophilic substrates by the corresponding methyltransferase via *in situ* formation of bisubstrate adducts, which should bind significantly more tightly with the enzyme than either substrate alone due to the synergistic binding interactions, thereby resulting in a more persistent complex, as illustrated in Scheme 2b. This is the premise of multisubstrate adduct inhibition,

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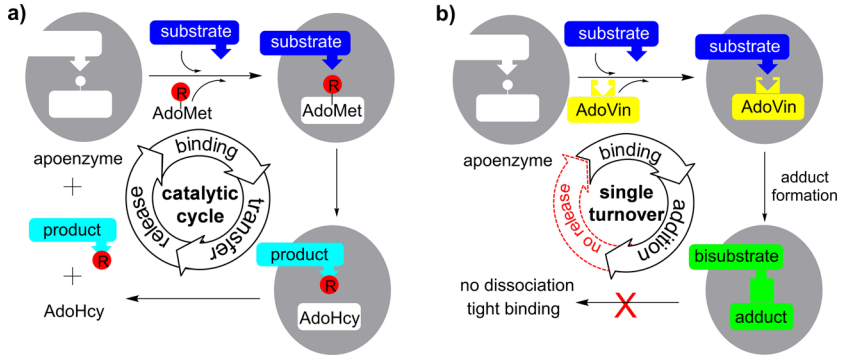
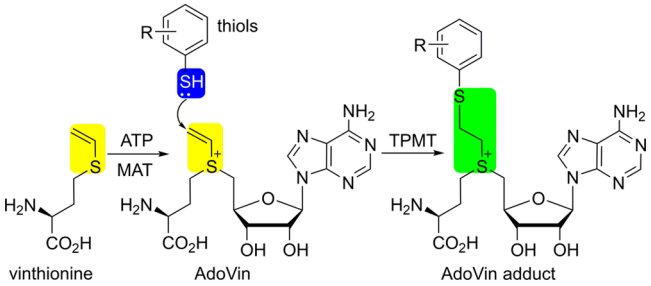
Scheme 2. (a) Transient Interactions between a Methyltransferase and Its Substrates or Products during the Catalytic Cycle and (b) Persistent Interaction (Tight Binding) between an Enzyme and the Bisubstrate Adduct Formed *in Situ*



championed by Coward, Pegg, and others.[3] This venerable concept has been explored—albeit in a few limited cases—to identify unknown enzymes and substrates, i.e., the formation of kinase-substrate complex via ATP-based cross-linker.[4] Weinhold, Rajski, Thompson, and others developed bisubstrate-adduct inhibitors for DNA and protein arginine methyltransferases (PRMTs) via AdoMet analogues with 5′-aziridinyl adenylates,[1a,2b,5] but to our knowledge, neither the tight binding between the adducts and MTases nor its application on identification of unknown substrates was discussed.

Our approach is demonstrated herein with *S*-adenosylvinthionine (AdoVin, Scheme 3), a new probe in which a vinyl

Scheme 3. Formation of AdoVin from Vinthionine and ATP-Catalyzed by MAT[a]



[a]TPMT-catalyzed *in situ* formation of bisubstrate adduct between AdoVin and thiol substrates.

sulfonium replaces the methyl sulfonium in AdoMet. Via an addition reaction to the vinyl sulfonium (a Michael-type acceptor), AdoVin and the nucleophilic substrate form a covalent tight-binding adduct.

As illustrated in Scheme 3, AdoVin was enzymatically synthesized from vinthionine and ATP catalyzed by methionine *S*-adenosyl transferase (MAT, or AdoMet synthetase, EC 2.5.1.6).[6] AdoVin shares similar characteristics with AdoMet (see Supporting Information, SI).

To examine the utility of AdoVin, thiopurine methyltransferase (TPMT, EC 2.1.1.67) with a broad specificity toward aromatic thiols was used (Scheme 3).[1e,2a] As listed in Figure 1, reduced Ellman's reagent (TNB, **a**) and other known substrates of TPMT all reacted with AdoVin and formed stable adducts depicted in Scheme 3, as confirmed by HPLC-UV–Vis and mass spectrometric analysis (see Figure S3.2.1–3.2.5). Conversely, nonsubstrates toward AdoMet (e.g., substituted phenols, **e**) did not react with AdoVin either. As such, the

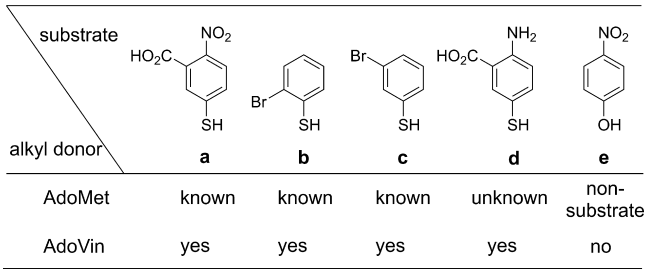


Figure 1. Same substrate specificity toward AdoVin and AdoMet.

substrate specificity toward AdoMet and AdoVin (the substrate surrogate) mirrors each other, thereby satisfying a critical requirement for such functional probes.

Next, tight binding of the resulting bisubstrate-adduct to the methyltransferase was investigated. Free ligands and enzyme-adduct complex were separated via either ultrafiltration or immobilized metal ion affinity chromatography (the recombinant TPMT contained a hexa-histidine-tag). Under both conditions, the AdoVin adducts were observed only in the enzyme complex (Figures 2 and Figure S3.3.1–3.3.3), indicating markedly tight binding between AdoVin adducts and MTases, as expected from synergistic interactions between the bisubstrate-adduct and the enzyme (Scheme 2b). Additionally, in the presence of vast excesses of competing reagents, AdoMet and AdoHcy, the adduct remains bound to the enzyme

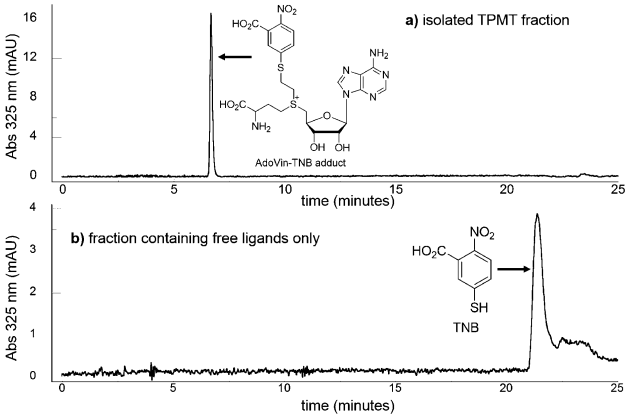


Figure 2. HPLC chromatograms (325 nm) of (a) methyltransferase-ligand complex and (b) fractions with only free ligands, showing that AdoVin-TNB adduct tightly bound with methyltransferase, while no dissociation of the AdoVin-TNB adduct was observed.

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(Figure S3.3.5). Moreover, the sulfonium ylide form of the adduct (Scheme S3.1.1), which closely mimics the neutral and linear transition state of the reaction, may exist when bound to the enzyme and thus contribute to the tight binding.

Adduct formation is both time dependent (first-order kinetics) and enzyme concentration dependent (Figure 3),

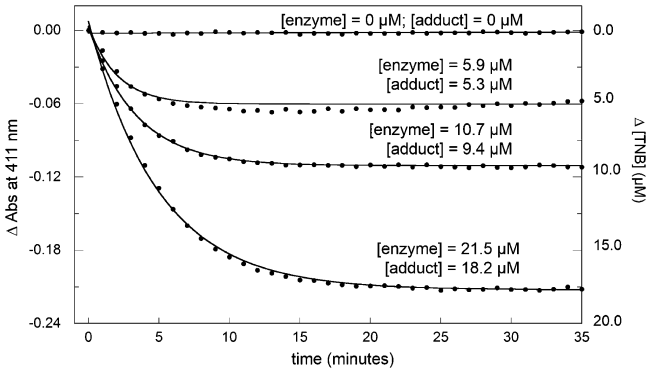

Figure 3. Changes in substrate concentration and TNB absorbance at 411 nm from the formation of adduct catalyzed by TPMT at various concentrations.

consistent with the proposed mechanism that involves a rapid initial binding of the thiol substrate and AdoVin with TPMT and the subsequent addition reaction. The first-order rate constant of adduct formation with AdoVin (k_{app} = 0.33 ± 0.12 min^{-1}), while the k_{cat} of transmethylation with AdoMet (13.6 ± 0.4 min^{-1}).[2a] In contrast, only a single turnover formation of the bisubstrate-adduct was observed (see Figures 3 and S3.4.2) while multiple turnovers were observed for transmethylation, again consistent with the markedly enhanced binding affinity of the bisubstrate-adduct compared to the individual components.

One of our goals is to identify unknown substrates of methyltransferases; in particular, no endogenous substrate for TPMT has been reported. To this end, AdoVin was incubated with the crude cell lysate of *E. coli* that expressed recombinant human TPMT, but no adduct was detected (see Figure S4.1.4), which was not unexpected as no aromatic thiol metabolites have been reported for *E. coli*. As a positive control, TNB was added to the crude cell lysate, and the corresponding adduct was detected, and again, only in the enzyme complex (see Figures 4 and S4.1.1−4.1.3), indicating tight binding under physiological conditions as well. Furthermore, Figure 4 illustrates the drastic enrichment of the adduct even from complex cellular mixtures. These experiments were also carried out in HeLa cell lysates with similar results (see Figure S4.4). It is worth noting that adduct formation was observed, even with competition from endogenous AdoMet which was present in the cell lysates.

Unexpectedly, in *E. coli* lysates, aside from the AdoVin-TNB adduct, another adduct (Figure 4) was detected in the TPMT complex, but only when TNB was added to the cell lysates, suggesting this unknown peak was derived from TNB. Based on the UV−Vis spectrum of the unknown adduct, the mass change (−30 Da), and fragmentation pattern of isotopic labeled adduct (see Figure S4.1.5),[7] we postulated that the nitro-group in TNB and adduct was reduced to an amine, which could be catalyzed by any of four nitroreductases existing in *E. coli*.[8] This assignment was confirmed by the authentic amino thiol (2-amino-5-mercaptobenzoic acid, AMBA, Figure 1, **d**) and the corresponding adduct with AdoVin (see Figure S4.2.1). It is

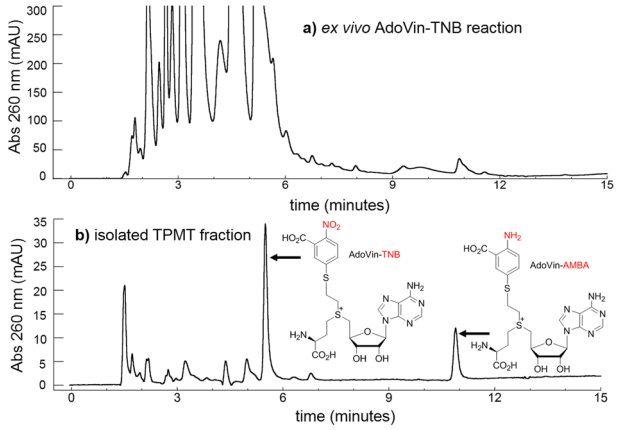

Figure 4. HPLC chromatograms (260 nm) of (a) *ex vivo* reaction of crude cell lysate and (b) the captured AdoVin-TNB and AdoVin-AMBA adducts from isolated TPMT complex, illustrating affinity enrichment.

worth noting that this amino thiol (AMBA) had not been reported as a substrate of TPMT but was confirmed in this work as a substrate toward AdoMet (see Figure S4.3.1). Altogether, this serendipitous finding underscores the utility of our approach in directly identifying enzyme substrates, even unknowns.

One general concern is whether AdoMet analogues modify enzymes. Using mass spectrometry, no modification was detected on either TPMT or MAT (Figure S5.2−5.3). While vinyl sulfonium is intrinsically reactive, extensive solvation of the highly charged sulfoniums in aqueous solution renders low reactivity.[6a,7c,9] For instance, no background reaction between thiols and AdoVin was observed (see Figure S3.1.4); and moreover, even under the *ex vivo* conditions where many metabolites exist, no adducts besides the expected ones were detected (see Figure S4.1.4).

In summary, our strategy can indeed capture and identify enzyme substrates, even unknowns. Conversely, if a substrate or methylation product is known, the corresponding enzyme can be identified as well. Applications in whole cells and organisms can also be envisioned, as AdoVin can be synthesized *in vivo* when vinthionine is supplemented.[6c−f,7c] Additionally, previous *in vivo* labeling with vinthionine resulted in modifications of a broad range of methyltransferases substrates (e.g., DNA and proteins), suggesting AdoVin was accepted as a substrate by other methyltransferases.[6c−f] Moreover, the formation of bisubstrate adducts may also have broad utility in facile generation of strong specific inhibitors and structural elucidation of substrate-enzyme interactions. Lastly, our approach can be applied toward many other enzymes, particularly those that catalyze group transfers.

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/jacs.5b05950.

Experimental details and data (PDF)

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Notes

The authors declare no competing financial interest.

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