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Very Important Paper

Identifying Unknown Enzyme–Substrate Pairs from the Cellular Milieu with Native Mass Spectrometry

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The enzyme–substrate complex is inherently transient, rendering its detection difficult. In our framework designed for bisubstrate systems—isotope-labeled, activity-based identification and tracking (IsoLAIT)—the common substrate, such as S-adenosyl-L-methionine (AdoMet) for methyltransferases, is replaced by an analogue (e.g., S-adenosyl-L-vinthionine) that, as a probe, creates a tightly bound [enzyme•substrate•probe] complex upon catalysis by thiopurine-S-methyltransferase (TPMT, EC 2.1.1.67). This persistent complex is then identified by native mass spectrometry from the cellular milieu without separation. Furthermore, the probe's isotope pattern flags even unknown substrates and enzymes. IsoLAIT is broadly applicable for other enzyme systems, particularly those catalyzing group transfer and with multiple substrates, such as glycosyltransferases and kinases.

In the complex cellular milieu, understanding which enzyme catalyzes which reaction is paramount for deciphering biology and disease. There are still many enzymes whose exact functions or substrates remain unknown. Conversely, we know the nature of some biotransformations but do not precisely know the responsible enzymes or proteoforms[1] (specific genetic and splicing variations, including any post-translational modifications). For example, more than 80 methyltransferases exist in humans, and whereas histone substrates are well characterized, non-histone substrates remain poorly understood.[2,3]

Screening is a common approach for identifying enzyme–substrate pairs.[4–6] For enzyme families with common substrates, such as S-adenosyl-L-methionine (AdoMet or SAM) for methyltransferases or adenosine triphosphate (ATP) for kinases, radiolabeled or affinity-labeled substrates, such as biotin-labeled ATP, or bio-orthogonally tagged analogues, such as propargyl and ketone AdoMet analogues, have been used to screen for enzyme–substrate pairs.[7–14] However, this methodology fails to identify specific enzyme–substrate pairs, particularly from cellular contexts, as multiple substrates can be tagged by multiple enzymes with no clear path for deconvolution.

Identifying enzyme–substrate pairs is hampered by the fact that their interactions are inherently transient. In a catalytic cycle (Figure 1 A), the enzyme binds the substrates, converts them to products, and releases them. With few exceptions,[15–18] ternary complexes are generally weakly bound and therefore cannot be directly observed by mass spectrometry (MS). However, non-catalytic complexes are observed by native MS. Enhancing the enzyme–substrate affinity is one key to successful detection.

Towards this end, we envisioned a new enzyme–substrate pair detection platform: isotope-labeled, activity-based identification and tracking, dubbed IsoLAIT. IsoLAIT is a method that links solution-phase activity to gas-phase detection by using a probe to capture, and native MS to identify, enzyme–substrate pairs from a single sample run with minimal sample preparation (Figure 1 B).

IsoLAIT has three keys: first, a probe that preserves the interaction between the enzyme and substrate for analysis; next, a detection method that retains enzyme–substrate interactions; and finally, a unique isotopic flag that clearly identifies the complex without a priori knowledge of its constituents.

First, the IsoLAIT probe forms an activity-based bisubstrate adduct (Scheme 1), which has sufficient affinity to the enzyme such that the [enzyme••substrate•probe] complex remains intact throughout subsequent analysis. Additionally, high affinity and low turnover enriches the [enzyme•substrate•probe] complex over the transient catalytic complex.

Second, the complex is analyzed by native MS, which maintains protein complexation in the gas phase. Augmented with nano-electrospray ionization, native MS has emerged as a powerful way to characterize protein–ligand binding.[18–20] Upon dissociation, the enzyme and substrate–probe are separated and can be further interrogated, elucidating the identity and structure of each component. As an added benefit, the complex is detected in multiple charge states (Figure 2), providing redundancy in complex systems where peaks overlap and helping to deduce the enzyme mass.

Third, the unique isotopic flag conferred by the probe is used as a telltale sign for the [enzyme•substrate•probe] complex. Overcoming the limitation of typical workflows that require pre-defined masses, we are able to identify unknown substrates and enzyme proteoforms with unforeseen modifications.

Herein, the IsoLAIT framework was demonstrated with methyltransferases, a large family of enzymes with diverse sub-

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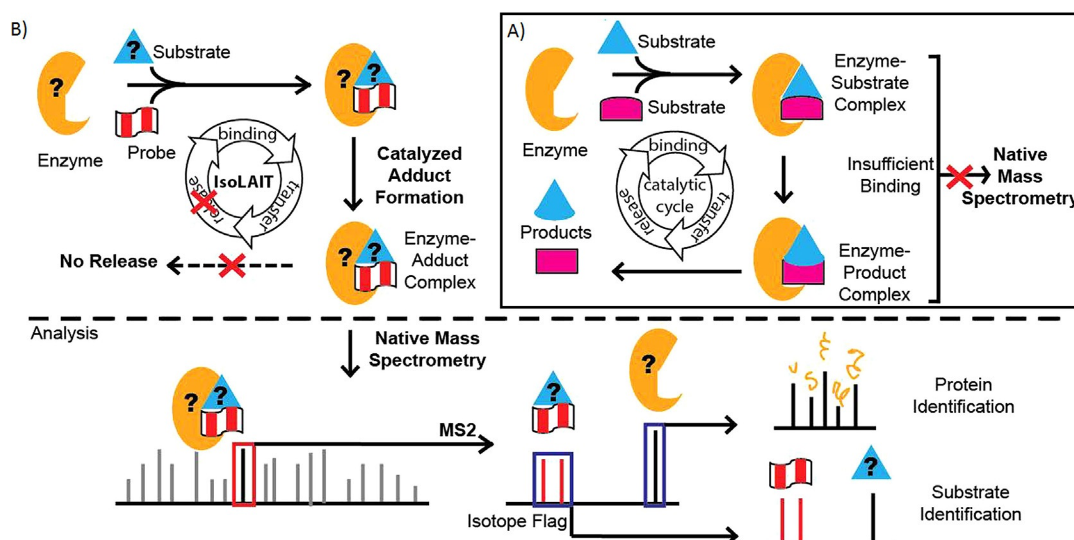
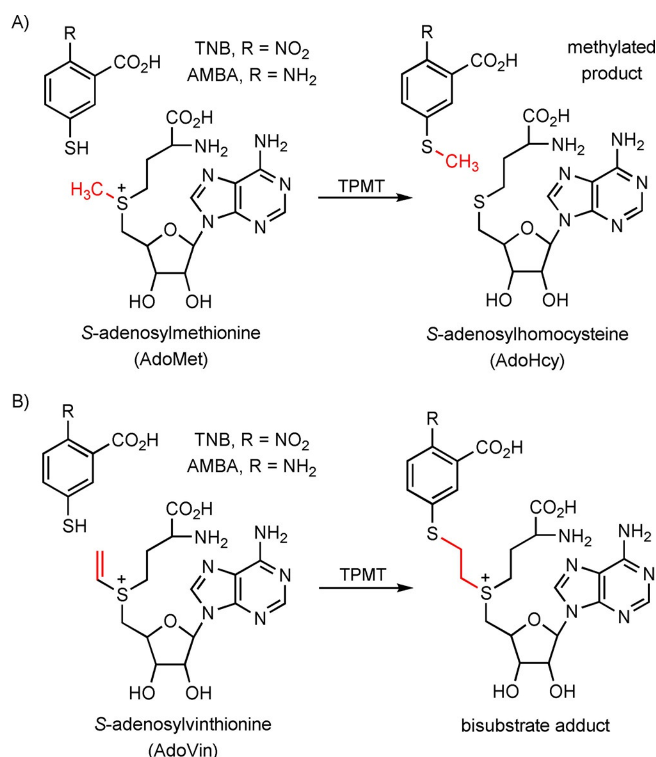


Figure 1. A) In the conventional catalytic cycle, an enzyme binds two substrates to create a ternary [enzyme•substrate•substrate] complex (inset). After catalysis, the [enzyme•product•product] complex dissociates, products are released, and the apo enzyme continues on to substrate binding. The transient enzyme–substrate interactions often do not have sufficient affinity to be analyzed by mass spectrometry. B) Alternatively, the IsoLAIT platform uses a substrate analogue as a probe. An enzyme catalyzes bisubstrate adduct formation between the probe and the enzyme's native substrate. The resulting tightly bound [enzyme•substrate•probe] complex can be analyzed by native mass spectrometry. Tandem mass spectrometry dissociates the enzyme from the adduct. The probe's isotopic flag is easily identified and serves as a reporter for such complexes; therefore, unknowns can be identified without a priori knowledge of the mass of the components involved. Further analysis can be used to elucidate the structures and sequences of the substrates and enzyme from the complex.



Scheme 1. A) AdoMet (or SAM) is used by thiopurine methyltransferase (TPMT) to methylate aromatic thiols and produce AdoHcy (or SAH). B) AdoVin, an AdoMet analogue and methyltransferase probe, forms a covalent bond with aromatic thiols under TPMT catalysis and results in a substrate–probe adduct that tightly binds to the enzyme.

strates. In transmethylation, the enzyme transfers a methyl from common donor AdoMet to a nucleophilic substrate, such as thiopurine methyltransferase[21] (TPMT, EC 2.1.1.67) which methylates thiophenols (Scheme 1 A). For this system, the selected IsoLAIT probe was *S*-adenosyl-L-vinthionine (AdoVin).[22,23]

We posited that TPMT would catalyze the formation of a substrate–probe adduct[7,24–29] (Scheme 1 B); indeed adduct formation between the substrate's thiol and the vinyl sulfonium in AdoVin was confirmed.[22] However, it was not clear whether such an [enzyme•substrate•probe] complex would bind sufficiently to survive native MS. To investigate, we prepared both in vitro and ex vivo (i.e., *Escherichia coli* cell lysate) samples (Figure S1 in the Supporting Information). AdoVin was prepared enzymatically[22] by using *S*-adenosyl-methionine synthetase (MAT, EC 2.5.1.6) with both labeled (+15 Da) and unlabeled ATP, along with vinthionine (Figure S1).

For the IsoLAIT platform, minimal sample preparation is required. The reaction mixtures were first exchanged into a volatile buffer near physiological pH (e.g., ammonium acetate adjusted to pH 8.0 with ammonium hydroxide) to maintain native conformations and enhance ionization and then were directly infused into the mass spectrometer. From the in vitro samples, the [enzyme•substrate•probe] complex and apo enzyme were readily detected and also resolved from each other (Figure 2 A, inset). Tandem MS (e.g., collision-induced dissociation (CID) at a collision energy of 500 eV) resulted in the apo enzyme and the substrate–probe adduct (e.g., TNB•AdoVin) (Figure 2 B). The latter was easily spotted from its signature 15 Da doublet, imparted by the isotopically labeled AdoVin probe (Figure 2 B, inset).

Further fragmentation of the TNB-AdoVin adduct was achieved by using higher collision energies for CID (1200 eV; Figure S2) or quasi-MS^3 analysis (Figure S3). The resulting frag-

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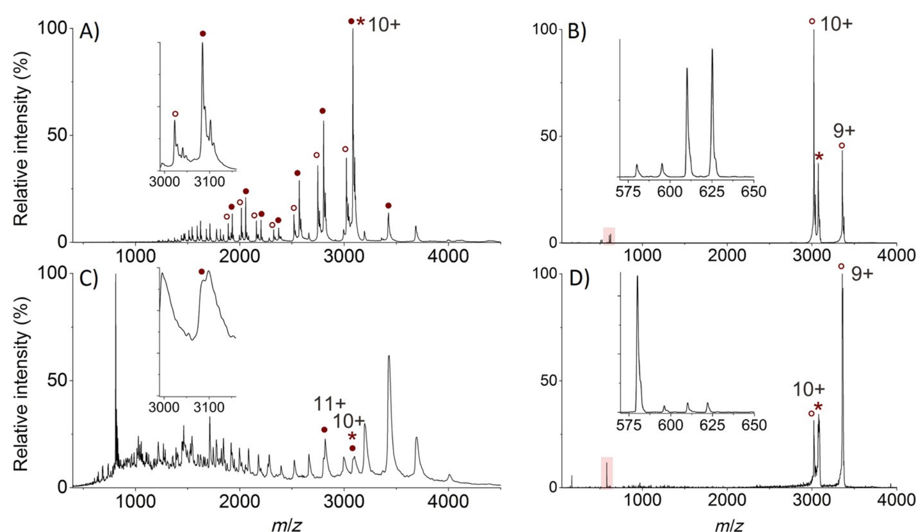

Figure 2. A) The in vitro [TPMT·TNB·AdoVin] complex (●) and apo TPMT (○) were detected in multiple charge states by native mass spectrometry. In this simple system, the bound and apo forms of TPMT were easily distinguished (inset). B) Collision-induced dissociation (CID) at a collision energy of 500 eV of the 10+ charged precursor ion of the [TPMT·TNB·AdoVin] complex (*) in (A). This collision resulted in apo TPMT with 10+ and 9+ charge states and the TNB-AdoVin adduct (highlighted and inset). Doublets observed in the TNB-AdoVin spectra are due to a mixture of natural and isotopically labeled AdoVin (+15 Da). C) The [TPMT·AMBA·AdoVin] complex (●) was identified in multiple charge states from whole cells without purification. In this complex matrix, bound and apo forms of TPMT were not resolved (inset). D) CID at a collision energy of 500 eV of the 10+ charged precursor ion of the [TPMT·AMBA·AdoVin] complex (*) in (C). After collision, multiple charge states for the apo enzyme were again observed, along with the AMBA-AdoVin adduct (highlighted and inset). In this case, the isotopically labeled probe was not used, resulting in a single peak. Isotopically labeled spectra were previously reported.[22]

ments confirmed the identity of the TNB substrate. As compared to the in vitro results, it is not clear whether both adduct-bound and apo forms of the enzyme from the ex vivo sample were present (Figure 2C, inset). This could be due to peak overlap, a common issue for the analysis of complex biological samples, as well as overall signal strength.

It is worth noting that the mass charge ratio for the ex vivo sample differed from that of the in vitro sample. Tandem MS of the ex vivo samples revealed the substrate–probe adduct to be unexpectedly modified. The nitro group of the TNB substrate was reduced to an amine (2-amino-5-mercaptobenzoic acid or AMBA) in the cell lysate, likely due to endogenous nitroreductases,[22,30] and the corresponding amine-containing substrate–probe adduct (i.e., AMBA-AdoVin) was formed (Figure 2D). Conventional workflows would likely miss this species, as the mass changes were unexpected; however, IsoLAIT is not bound by predefined masses but screens for mass patterns imparted by the probe and thus will identify substrates no matter their modifications.

Equally interesting is the ability to identify the functional enzyme proteoform. For tandem mass spectra, identifying the apo and adduct-bound-enzyme peaks was straightforward. Upon closer analysis of apo-TPMT, the observed and theoretical masses differed; the enzyme underwent methionine cleavage when expressed in *E. coli* (Figure S4). These results highlight IsoLAIT's utility in identifying an unknown, biologically relevant, active proteoform.

Complex dissociation by surface-induced dissociation (SID) instead of CID yielded similar results (Figure S5). Though redundant in the case of the monomeric TPMT enzyme, as interest in characterizing multimers or protein complexes increases,[20] the utility of SID is significant.[31,32]

Throughout screening, we attempted to identify the enzyme–substrate complex without the use of a probe. Though TPMT was observed in complex with the common substrate (AdoMet) and by-product *S*-adenosylhomocysteine (AdoHcy or SAH; Figures S6 and S7), we did not observe the TPMT enzyme bound to either TNB or AMBA substrates without the use of the AdoVin probe, again highlighting the transient nature of substrate–enzyme interactions.

Through the given examples, IsoLAIT demonstrated its robustness as a general platform, succeeding in contexts where conventional workflows might have failed. Peak overlap in complex matrices was overcome by using the probe as a flag in the tandem mass spectra. Importantly, this method requires no prior knowledge of the specific enzyme or substrate for identification. The unexpected alteration of substrate observed in the ex vivo samples perfectly illustrate both the challenges associated with biological systems and the utility of our methods. Had the ex vivo screening been based on the in vitro results, we would have had completely missed the [enzyme·substrate·probe] complex from ex vivo samples, due to modifications to both the enzyme and substrate.

What we have demonstrated is a streamlined version of IsoLAIT. The framework could be augmented by including other compatible separation techniques providing even greater detail. Upstream sample purification, inline liquid chromatography methods, and downstream separation, such as ion mobility, which we have successfully used, could further enhance the method.

The IsoLAIT framework solves two key challenges in detecting enzyme–substrate pairs: first, how to overcome the transient nature of enzyme–substrate interactions, and second, how to identify components from a complex mixture with unex-

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pected modifications. The former (transient interaction) is overcome by the combination of a probe that perpetuates enzyme–substrate interactions and native MS that retains binding in the gas phase. The latter (identification) is accomplished by using an activity-based probe and monitoring the telltale isotopic pattern.

This appears to be the first reported example of mass spectrometric identification of enzyme–substrate complexes from the cellular milieu, but it need not be the last. In this case, we used a TPMT-specific probe (i.e., AdoVin), but IsoLAIT probes can be tailored for other systems. More promiscuous probes could be used to screen for enzyme–substrate pairs more broadly. For example, aziridinoadenosines have been shown to work as activity-based probes for a range of methyltransferases and form stable, tight-binding bisubstrate adducts.[26,33–35] Previous studies with chemical tagging and shotgun proteomics might be amenable for IsoLAIT adaptation.[36,37] IsoLAIT can be broadly applicable and similarly successful for other enzyme systems, particularly those catalyzing group transfer and with multiple substrates, such as glycosyltransferases and kinases.

Experimental Section

General procedures: All chemicals were reagent purity or higher and were purchased from Sigma and Fisher unless otherwise noted. Immobilized metal ion affinity chromatography (IMAC) was performed on HisTrap HP columns. Ultrafiltration was carried out by using filters with a 10000 molecular weight cut-off (MWCO).

Preparation of *S*-adenosylvinthionine (AdoVin): *S*-Adenosyl-L-vinthionine [CAS 83768-89-2] was prepared enzymatically[22] by using *S*-adenosyl-methionine synthetase (MAT, EC 2.5.1.6) with both labeled (+15 Da) and unlabeled ATP (10 mM), along with vinthionine (1 mM). The reaction proceeded in a potassium phosphate buffer (50 mM, pH 8.0) with KCl (5 mM), and $MgCl_2$ (2.5 mM) and was initiated with MAT (50 μM) and incubated at 37 °C. After 2–4 h incubation, this mixture was used as the in situ probe. AdoVin has similar stability to AdoMet; thus, the samples were used immediately and without freezing.

In vitro reactions: For the in vitro samples, His-tagged TPMT was grown in transformed *E. coli* and purified on an IMAC column.[38] Adduct formation was performed in potassium phosphate (50 mM, pH 8.0). The reaction solution contained the in situ AdoVin probe at 1 mM, 2-nitro-5-thiobenzoic acid (TNB; 500 μM), tris(2-carboxyethyl)phosphine (TCEP; 2 mM), MTAN (3.5 μM) and TPMT (100 μM). The reaction was incubated at 37 °C for 3 h.

Ex vivo reactions: For ex vivo reactions, TPMT-transformed *E. coli* were grown at 37 °C in LB broth, stimulated with isopropyl β-D-1-thiogalactopyranoside (IPTG; 1 mM) for 12 h, then washed three times with lysis buffer (50 mM potassium phosphate, 0.5 M NaCl, pH 8.0). Cells were lysed by sonication on ice, and the cell debris and unbroken cells were removed by centrifugation (8000 *g*, 4 °C, and 60 min). Exogenous reagents were added to the supernatant: in situ AdoVin probe (1 mM), TNB (500 μM), TCEP (2 mM), MTAN (3.5 μM). The mixture was allowed to react at 37 °C for 3 h.

Sample preparation: Prior to analysis by MS, all samples were exchanged into ammonium acetate buffer (20 mM, adjusted to pH 8.0, with ammonium hydroxide) by using at least ten cycles of concentration and dilution in a 10 kDa MWCO centrifuge and ultrafiltration concentrator. Samples were then frozen at −80 °C and thawed immediately prior to analysis. Note: in the case where freezing denatures or unfolds the proteins, samples should be stored at 4 °C or analyzed immediately. The sample was further diluted in ammonium acetate (20 mM; pH 8). The concentration of ex vivo [TPMT·AMBA·AdoVin] was estimated at 2 μM, as determined by SDS-PAGE. The concentration of the in vitro [TPMT·TNB·AdoVin] was 10 μM, as determined by A280nm.

Native mass spectrometry: The nano-electrospray experiments were performed on a Synapt G2S HDMS (Waters Corporation, Wilmslow, UK) with a customized surface-induced dissociation (SID) device installed before the ion mobility cell as previously described.[32] Each sample was added to a glass capillary pulled by using a Sutter Instruments P-97 micropipette puller (Novato, CA) and electrically connected to high voltage with a platinum wire. The nano-electrospray source was at a voltage of 1.2–1.5 kV. The sampling cone voltage was set to 20 V, and the source offset voltage was set to 20 V to avoid source activation of the complex. Other instrument conditions were 5×10^{-3} mbar for the source pressure, 2.0 mL min^{-1} gas flow rate to the trap cell, 120 mL min^{-1} gas flow to the helium cell, and 60 mL min^{-1} gas flow to the ion mobility cell. The ion mobility wave velocity was 200 m s^{-1}, and the wave height was 16 V. The time-of-flight (ToF) analyzer pressure was 1.2×10^{-6} mbar.

Tandem mass spectrometry: Tandem mass spectrometry experiments were performed by dissociation of the selected ions with CID and SID. CID experiments were conducted with a trap gas flow rate of 4.0 mL min^{-1}, and SID was conducted with a trap gas flow rate of 2.0 mL min^{-1}. The CID and SID (MS^2) experiments were conducted in the trap travelling wave ion guide region before the ion mobility cell. The acceleration voltage in CID and SID was obtained as described previously.[31,32] The collision energy in eV was calculated by multiplying the acceleration voltage by the charge state of the precursor ion. The fragment ions generated from MS^2 experiments were separated in the ion mobility cell. The quasi MS^3 experiment was conducted by selection in the quadrupole, activation in the trap CID cell, separation in the ion mobility cell, and activation in the transfer traveling wave ion guide.

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