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# Fluorescent Probes for Monitoring Serine Ubiquitination

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## Abstract

In a radical departure from the classical E1–E2–E3 three-enzyme mediated ubiquitination of eukaryotes, the recently described bacterial enzymes of the SidE family of *Legionella pneumophila* effectors utilize $NAD^+$ to ligate ubiquitin onto target substrate proteins. This

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Supporting Information

The Supporting Information is available free of charge at https://pubs.acs.org/doi/10.1021/acs.biochem.0c00067.
Materials and Methods, Figures S1–S6, and Tables S1 and S2 (PDF)

Accession Codes

SdeA, Q5ZTKA; SidJ, Q5ZTK6; DupB, Q5ZSK8; ubiquitin, P0CG48; calmodulin, P0DP23.

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outcome is achieved via a two-step mechanism involving (1) ADP ribosylation of ubiquitin followed by (2) phosphotransfer to a target serine residue. Here, using fluorescent $NAD^+$ analogues as well as synthetic substrate mimics, we have developed continuous assays enabling real-time monitoring of both steps of this mechanism. These assays are amenable to biochemical studies and high-throughput screening of inhibitors of these effectors, and the discovery and characterization of putative enzymes similar to members of the SidE family in other organisms. We also show their utility in studying enzymes that can reverse and inhibit this post-translational modification.

The post-translational modification of proteins with the 76-residue modifier ubiquitin (Ub) is a vital process in eukaryotic cells.[1] Ubiquitination, catalyzed by the E1–E2–E3 three-enzyme cascade and using ATP as a cofactor, is involved in protein recycling, DNA repair, and immunity. Most eukaryotic proteins undergo this modification at some point in time, highlighting its importance in proper cellular function. A rapidly evolving area of research pertains to the interactions of bacterial effectors with the host ubiquitin system.[2] Prokaryotic organisms notably lack ubiquitin systems. However, several pathogenic bacteria have evolved a variety of strategies to effect the ubiquitin signaling of their eukaryotic hosts, such as mimics of eukaryotic ubiquitin-interacting proteins as well as novel motifs of their own.[3] A striking example of the latter is the unusual $NAD^+$-dependent ubiquitination of substrates by the SidE family of *Legionella pneumophila* effectors. The SidE family is comprised of four large (>150 kDa) modular proteins: SdeA, SdeB, SdeC, and SidE. During infection, these and other proteins are trans-located into the host cell by way of the Dot/Icm type IV secretion system of the bacteria. These proteins are noteworthy in that they have been found to carry out the first example of protein ubiquitination occurring via a mechanism outside of the canonical eukaryotic pathway.[4] This process, using $NAD^+$ instead of ATP and requiring only one protein instead of three, results in a ribose–phosphate linkage between Arg42 of Ub and a Ser residue of the substrate.[5,6] This stands in contrast to the standard isopeptide linkage between the C-terminus of Ub and a Lys residue of the substrate observed in typical ubiquitination. Along with this ubiquitin-ligating activity, the SidE proteins can generate phosphoribosylated ubiquitin as a byproduct (Ub-PR). Mechanistically, this ubiquitination reaction is initiated by a mono-ADP-ribosyltransferase (mART) reaction of Arg42 of Ub (catalyzed by the mART domain), resulting in the intermediate Ub-ADP-ribose (Ub-ADPR). This molecule undergoes a subsequent phosphotransferase reaction [catalyzed by the phosphodiesterase (PDE) domain] to be linked onto a substrate Ser (and possibly other hydroxyl-containing) residue or simply to be hydrolyzed into Ub-PR, mechanistically explained as a phosphotransfer to water (Figure 1).[7] Interestingly, another *Legionella* effector, SidJ, has been found to be able to block SidE activity by glutamylating their catalytic residues, acting as a regulator of this process.[8–11] Recently, two other enzymes, homologous to the PDE domain, called DupA and DupB, were also found to regulate SidE-catalyzed ubiquitination. Instead of blocking catalysis, however, they remove phosphoribosyl-linked Ub from substrate proteins, reversing the modification.[12,13] These two regulatory mechanisms highlight a remarkable complexity to this new post-translational modification.

SidE proteins are therefore of great interest to study, especially considering the fact that they are required for optimal *Legionella* virulence.[4] The discovery of this new post-translational modification also demands an investigation into whether this process occurs naturally outside of *Legionella* infection. However, bioinformatics-based identification of homologues has been challenging because of the substantial divergence of the sequence of these effectors from known enzymes. To this end, we have developed continuous, fluorometric assays to measure both steps of this reaction in a sensitive and high-throughput manner. For the first step, we show the utility of two disparate emissive analogues of $NAD^+$ (1) in our assays; the classical nicotinamide 1,N6-ethenoade-nine dinucleotide ($\varepsilon NAD^+$) (2)[14] as well as the recently developed $N^{tz}AD^+$ (3) that is based on an isothiazolo[3,4-*d*]pyrimidine core (Figure 2a).[15] To monitor the crucial second step, we have synthesized a peptide that behaves as a substrate, measuring ubiquitination by fluorescence polarization. These techniques have yielded new insights into the biochemistry of SidE effectors and will prove useful for future attempts of inhibitor screening and discovery/characterization of new members of this enzyme class.

It has been shown that the activity of canonical mART enzymes can be measured with the aforementioned $NAD^+$ analogues, due to the fact that these molecules are internally quenched by the nicotinamide moiety.[16] A loss of the nicotinamide from $\varepsilon NAD^+$ or $N^{tz}AD^+$ resulting from mART activity will therefore result in a conspicuous increase in fluorescence. To test whether the ADP ribosylation of Ub can be similarly measured, we performed this assay utilizing four constructs of SdeA, a representative SidE family protein. We used the full length protein ($SdeA_{FL}$), the ubiquitinating construct spanning residues 181–1000 ($SdeA_{181–1000}$), the mART construct ($SdeA_{519–1100}$), and a mutant of the full length protein that cannot perform the mART reaction with catalytic residues Glu860 and Glu862 mutated to Ala ($SdeA_{E/A}$). Incubation with $\varepsilon NAD^+$ and Ub resulted in an increase in fluorescence emission at 410 nm, which could be observed in real time (Figure 2b). Interestingly, we observed that $SdeA_{FL}$ was the most active construct of the three, and $SdeA_{519–1100}$ was the least active. This effect is in line with previous studies[17] and is possibly a result of the coiled-coil (CC) domain stabilizing the productive orientation of the mART domain. These results were also observed when $N^{tz}AD^+$ was used (Figure S1). The fluorogenic intermediate Ub-$\varepsilon$ADPR was also isolated and analyzed by LC-MS to verify its identity (Figure S2). Together, these data demonstrate the utility of fluorescent $NAD^+$ analogues in studying the first step of SidE-catalyzed ubiquitination.

While the assay described above is useful for monitoring ADP ribosylation of Ub, it is limited due to the fact that the second step of the reaction, substrate serine ubiquitination, cannot be tracked with this method. Indeed, further studies have identified substrate ubiquitination as the key step that is pertinent to *Legionella* pathogenesis.[17] A mutant of SdeA deficient in catalyzing step 2 while still capable of catalyzing step 1 was unable to rescue normal growth in a SidE-deficient strain. Thus, it is important to assay this process quantitatively and develop probes to further understand the mechanism and substrate selection of these enzymes. To this end, a model substrate was synthesized. Previous studies have suggested that SidE proteins target serine residues on unstructured, flexible regions of proteins, such as the N-terminus of many Rab GTPases.[18] We therefore synthesized a peptide consisting of the first nine residues of Rab1, a known SidE substrate with a 5′-

fluorescein group on the amino terminus. These N-terminal residues are unresolved in the crystal structure of Rab1, indicating that they are likely unstructured (Figure 3). To confirm that this peptide behaved as a ubiquitination substrate, sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) analysis revealed the presence of a fluorescent band around 10 kDa upon reaction with Ub, SdeA, and $NAD^+$. In the absence of $NAD^+$, this band was not observed (Figure 4b).

We then attempted to track the ubiquitination of the peptide by SdeA in real time. Due to the approximately 10-fold size difference between the peptide and the peptide–Ub conjugate, we anticipated a fluorescence polarization (FP) increase as the peptide was ubiquitinated by SdeA. When a reaction mixture containing $SdeA_{181–1000}$, Ub, $NAD^+$, and a peptide was subjected to FP measurement ($\lambda_{ex}$ = 485 nm; $\lambda_{em}$ = 528 nm), the conversion of the peptide to the Ub–peptide conjugate was observed via a significant increase in FP as expected (Figure 4 and Figure S2b). A Michaelis–Menten analysis with respect to the peptide produces an apparent $K_M$ of ~80 $\mu$M and a $k_{cat}$ of ~1.6 $s^{-1}$ (Figure S6).

Because our fluorescent peptide was derived from Rab1, it contained two Ser residues at positions 2 and 3. To determine whether both residues or only one was targeted by SdeA, analogous peptides were synthesized with the respective Ser residue mutated to Ala (peptides MSA and MAS). Intriguingly, we found that while peptide MAS retained activity similar to that of the original peptide (MSS), peptide MSA was not ubiquitinated by SdeA. To further explore the substrate selectivity of SdeA toward other hydroxyl-containing residues, two additional peptides were synthesized with Thr or Tyr at position 3 (peptide MAT or MAY, respectively). Neither peptide was found to be ubiquitinated by SdeA (Figure 4f). This result suggests that SidE enzymes recognize serine specifically, and that the positioning of Ser plays a role in substrate recognition. Further structural studies may provide additional insight into the basis of this selectivity. Due to the importance of the SidE family of enzymes in *Legionella* virulence, and their conservation among a wide variety of *Legionella* species, they may comprise a new therapeutic target.

The aforementioned assay is a facile technique for screening and characterizing inhibitors for this new enzyme class. A previous study suggested that adenosine monophosphate (AMP) could serve as a weak inhibitor of SdeA-catalyzed ubiquitination.[17] Utilizing our assay, incubation with AMP or ADP-ribose resulted in the impairment of peptide ubiquitination (Figure 4h).

The regulation of phosphoribosyl ubiquitination (PR ubiquitination) by other *Legionella* effectors has recently attracted considerable interest. To control the levels of PR-linked ubiquitin, at least two systems have recently been discovered. First, the enzyme SidJ is a glutamylase that covalently modifies the catalytic E860 of SdeA, effectively switching it off.[8–11] The inhibitory effect of SidJ is manifest only when it is bound to the host calmodulin. To study the activity of SidJ, we reacted SdeA with the SidJ–calmodulin complex and observed a striking, time-dependent decrease in SdeA ubiquitinating activity (Figure 5a and Figure S3a). By plotting the initial rates of our SdeA samples over time, we were able to generate a progress curve for SidJ-catalyzed modification of SdeA (Figure S3b).

Furthermore, the recently reported discovery of *Legionella* enzymes that reverse PR-linked serine ubiquitination catalyzed by the SidE family has intriguingly added a new layer of regulation to this post-translational modification. These enzymes, named DupA and DupB (from lpg2154 and lpg2509, respectively), remove phosphoribosyl-linked ubiquitin from substrates (Figure S3c,d).[12,13] Their catalytic action and their structure closely resemble that of the PDE domain of SidE proteins. It is possible that these regulatory effectors exist to prevent uncontrolled ubiquitination of substrates by SidE, as deletion of DupA and DupB caused the accumulation of PR-ubiquitinated proteins in infected cells.[12,13] We tested whether we can monitor the deubiquitination of PR-linked ubiquitin from our synthetic fluorogenic peptide substrate. A two-step assay was performed where peptide ubiquitination was followed by incubation with DupB (also known as SdeD). An increase in FP followed by a marked decrease to baseline levels upon addition of DupB was observed (Figure 5b). Also, in the place of purified DupB, incubation with the lysate of a strain of *L. pneumophila* lacking the SidE family also resulted in a decrease in FP to baseline levels (Figure 5c). This further highlights the application of this assay toward probing cell lysates to study regulators of SidE enzymes.

The study of serine ubiquitination catalyzed by the SidE family of bacterial effectors has highlighted an elegant new mechanism of post-translationally modifying host proteins. We have developed a robust, real-time method for studying this process via fluorescence, including mutation and inhibition studies that have previously been performed via gel electrophoresis-based end point analysis. We have also elucidated the position and residue selectivity of SidE enzymes, where future work will be necessary to determine the structural basis for serine and positional preference. In addition, it remains to be seen whether serine ubiquitination via the SidE mechanism exists in organisms outside of *Legionella*. Our method provides a useful tool for discovering new enzymes that can either catalyze or regulate this process.

## Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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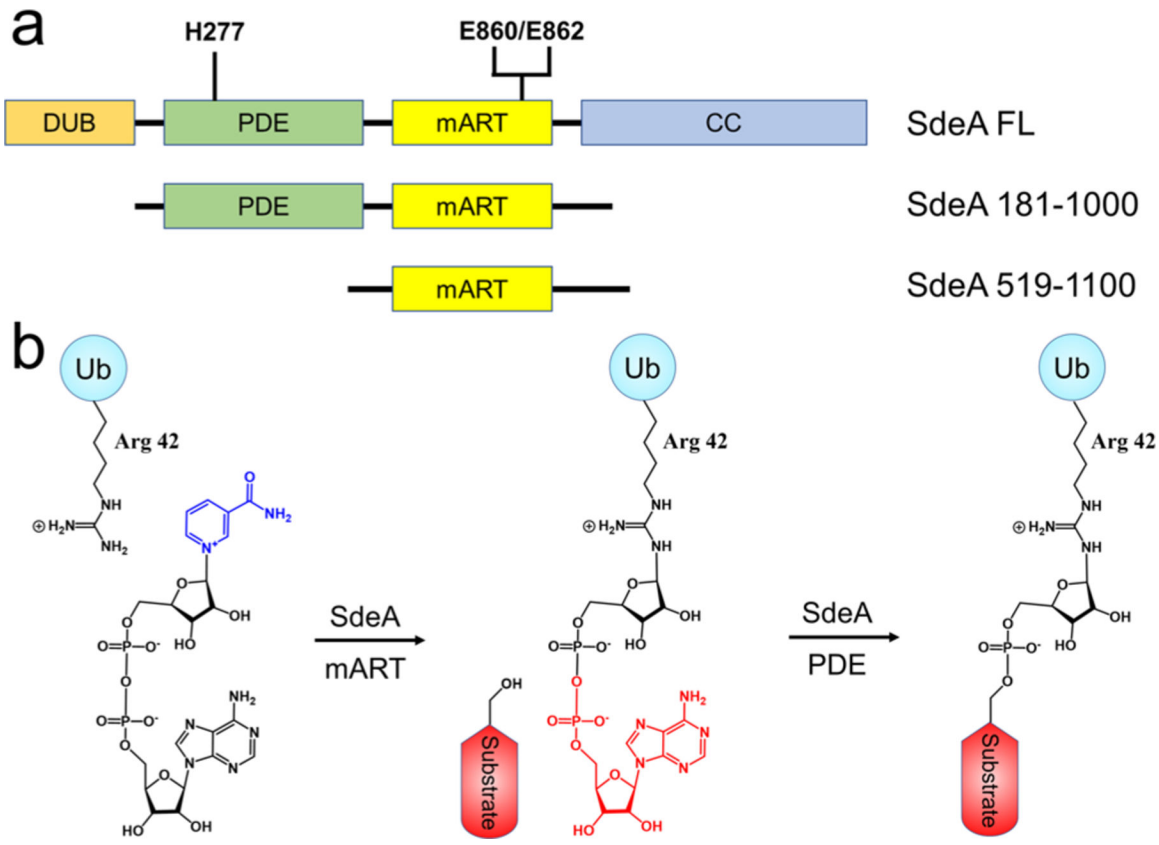


**Figure 1.**

General features of SdeA. (a) Three key constructs were utilized in this study: full length enzyme, a construct containing both PDE and mART domains, and a construct containing only the mART domain. (b) Overall SidE mechanism of action that involves first mono-ADP ribosylation of ubiquitin at Arg42, catalyzed by the mART domain. Next, this ADP-ribosylated intermediate reacts with the PDE domain to be transferred to a Ser residue of the substrate protein.

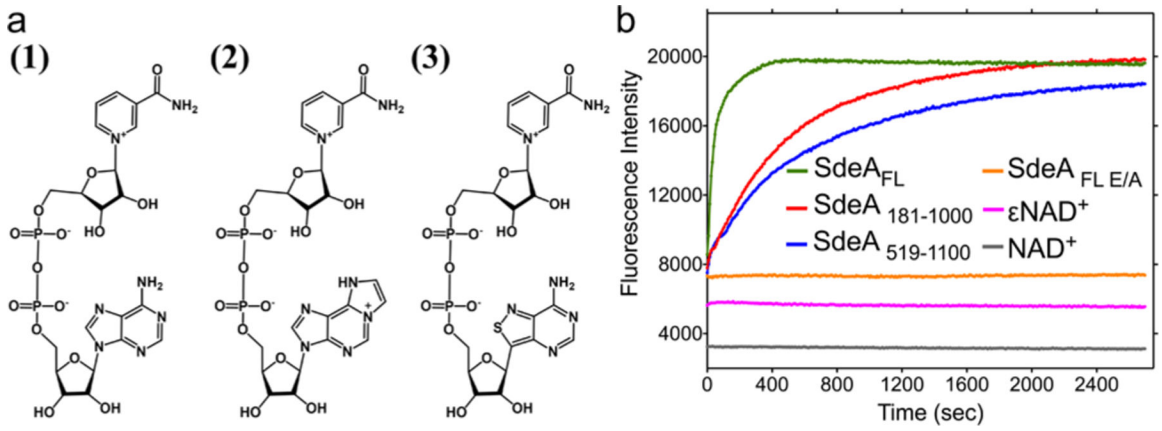


**Figure 2.**

Fluorescent $NAD^+$ analogues used for monitoring the first step of SdeA-catalyzed ubiquitination. (a) Structures of $NAD^+$, $\varepsilon NAD^+$, and $N^{tz}AD^+$. (b) When SdeA and Ub are incubated with $\varepsilon NAD^+$, a marked increase in fluorescence is observed in a time-dependent manner, consistent with the liberation of the nicotinamide group and loss of quenching. The activities of three distinct constructs of SdeA are compared.

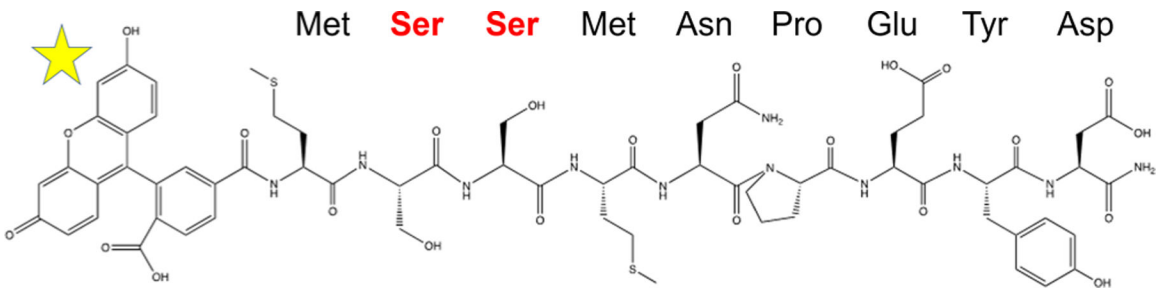


**Figure 3.**

Structure of the synthetic peptide substrate for SdeA ubiquitination assays. A nine-residue peptide with an N-terminal fluorescein group was designed, derived from the N-terminus of the known ubiquitination substrate protein Rab1.

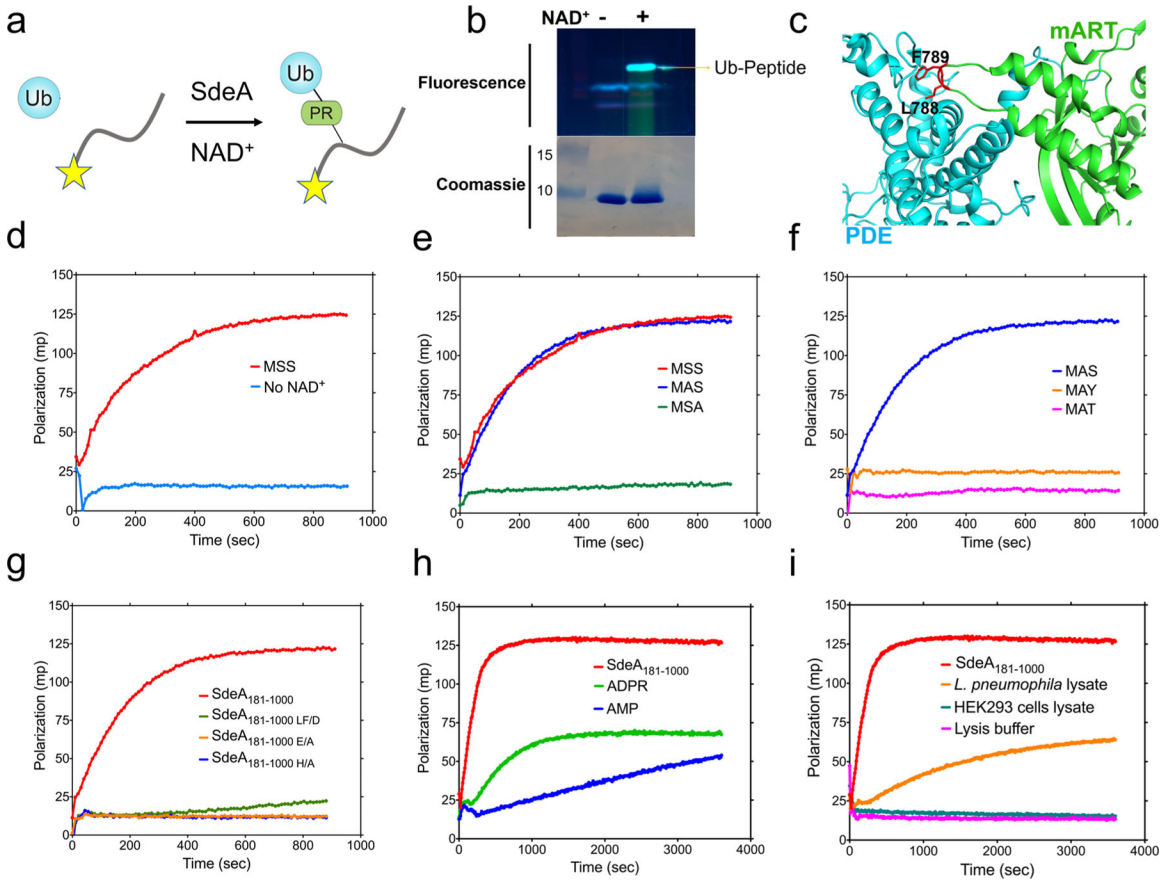


**Figure 4.**

(a) Overall scheme of a ubiquitination assay. (b) Fluorescence imaging of SDS–PAGE analysis of SdeA reaction in the presence of the peptide substrate reveals a fluorescent band when $NAD^+$ is included, indicative of successful reaction. (c) Crystal structure of SdeA mART and PDE domains showing interdomain interactions, the importance of which can be tested via this fluorometric assay (Protein Data Bank entry 5ZQ2).[18] (d) Including the fluorescent Rab1 peptide in the SdeA reaction caused a significant increase in FP. (e) Selective mutation of the Ser residues in the peptide substrate shows that the third Ser is likely targeted. (f) Mutation of the Ser to other hydroxyl-containing residues, such as Thr or Tyr, causes activity to be lost, indicating that SdeA specifically targets Ser residues. (g) Analysis of SdeA mutants using the fluorometric assay allows us to compare their activity in real time. The catalytic mutants (E/A and H/A) as well as the intradomain binding mutant (SdeA LF/D) were utilized. (h) Analysis of inhibition of SdeA-catalyzed ubiquitination. The nucleotides ADPR and AMP were included in the reaction mixture, resulting in inhibition of peptide ubiquitination. (i) Substituting cell lysates for purified SdeA protein allows this assay to be used to detect ubiquitination of the synthetic peptide. The *L. pneumophila* lysate caused an increase in the level of polarization. Within the parameters of this assay, the HEK cell lysate did not show a detectable increase.

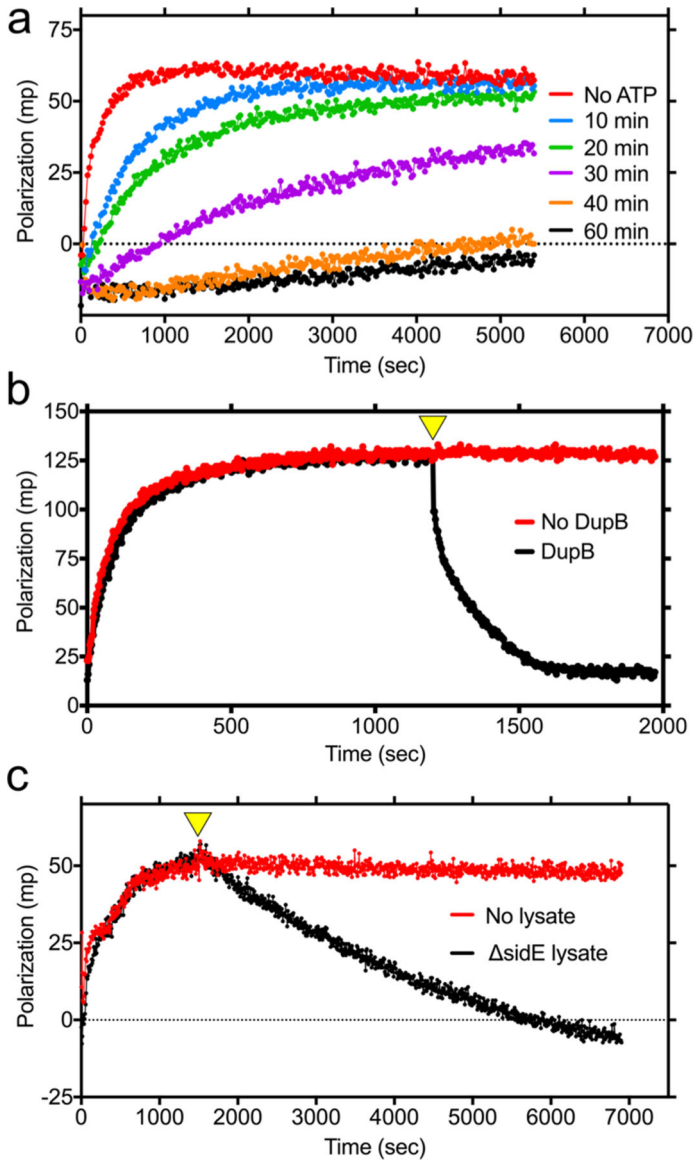


**Figure 5.**

(a) Analysis of SidE regulators. SidJ was preincubated with SdeA for the indicated time points, and ubiquitination was measured. (b) Purified DupB (SdeD) was added to the preubiquitinated peptide, demonstrating removal of PR-linked Ub. (c) The *L. pneumophila* lysate without SidEs was also utilized in the two-step assay described above to show Dup activity.