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Allosteric Inhibition of the NS2B-NS3 Protease from Dengue Virus

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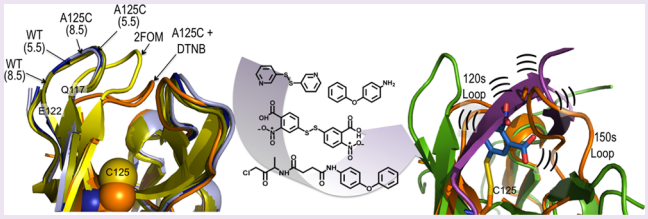
Supporting Information

ABSTRACT: Dengue virus is the flavivirus that causes dengue fever, dengue hemorrhagic disease, and dengue shock syndrome, which are currently increasing in incidence worldwide. Dengue virus protease (NS2B-NS3pro) is essential for dengue virus infection and is thus a target of therapeutic interest. To date, attention has focused on developing active-site inhibitors of NS2B-NS3pro. The flat and charged nature of the NS2B-NS3pro active site may contribute to difficulties in developing inhibitors and suggests that a strategy of identifying allosteric sites may be useful. We report an approach that allowed us to scan the NS2B-NS3pro surface by cysteine mutagenesis and use cysteine reactive probes to identify regions of the protein that are susceptible to allosteric inhibition. This method identified a new allosteric site utilizing a circumscribed panel of just eight cysteine variants and only five cysteine reactive probes. The allosterically sensitive site is centered at Ala125, between the 120s loop and the 150s loop. The crystal structures of WT and modified NS2B-NS3pro demonstrate that the 120s loop is flexible. Our work suggests that binding at this site prevents a conformational rearrangement of the NS2B region of the protein, which is required for activation. Preventing this movement locks the protein into the open, inactive conformation, suggesting that this site may be useful in the future development of therapeutic allosteric inhibitors.



Dengue virus (DENV) is the causative agent in dengue fever, dengue hemorrhagic disease, and dengue shock syndrome. All four serotypes of dengue virus are transmitted to humans by the *Aedes aegypti* and *Aedes albopictus* mosquitoes.[1−6] Globally nearly 2.5 billion people are at risk of dengue virus infection and over 100 million infections are reported annually.[6] Dengue virus infection also causes 22,000 deaths each year in areas where it is endemic.[7] Despite intensive biomedical studies, no vaccine nor drug has been approved to date.[1,8−10]

Dengue virus contains a positive-strand RNA genome that can be directly translated into a single polyprotein chain by host-cell translation machinery. The polyprotein precursor comprises three structural proteins, capsid (C), membrane (M), and envelope (E), and five nonstructural proteins, NS1−5. In the polyprotein precursor the proteins are arranged as NH_2-C-prM-E-NS1-NS2A-NS2B-NS3-NS4A-NS4B-NS5-COOH.[11−13] Maturation of the precursor is essential for the viral life cycle and requires post-translational proteolytic processing by the dengue virus protease (NS2B-NS3pro) to liberate individual viral proteins from the polyprotein chain. NS2B-NS3pro, a trypsin-like serine protease, performs the post-translational proteolytic processing of the precursor.[14,15] NS2B-NS3pro cleaves the viral protein at the NS2A/NS2B, NS2B/NS3, NS3/NS4A, and NS4B/NS5 junctions, as well as in the capsid protein.[16,17] Full processing releases the viral protease (NS2B-NS3pro), helicase (NS3),[18−20] methyltransferase (NS5), and RNA-dependent RNA polymerase (NS5).[21] The other nonstructural proteins such as NS1 and NS2 are involved in the viral replication complex, while NS4 is an integral membrane protein that plays a role in induction of membrane alteration.[22−24]

It is clear that the flavivirus proteases including NS2B-NS3pro are essential for viral replication and infectivity, as shown by a number of previous studies. For example, yellow fever virus genetically modified to contain only inactive NS2B-NS3pro is unable to infect target cells.[25] Similarly, treatment of cells with a peptide that inhibits NS2B-NS3pro decreases dengue virus infection by 80%.[26] In addition, inhibition of viral proteases is a well-established route for preventing viral infection in clinical settings. A large number of HIV protease inhibitors are clinically used to treat HIV infection and AIDS (for a review, see ref 27). Two hepatitis C virus protease inhibitors recently approved for therapeutic use have likewise become the standard of care.[28] Thus one of the most prominent attempted strategies for dengue virus therapy has been development of small compounds that target the active site NS2B-NS3pro.[29−33]

NS2B-NS3pro has a catalytic triad (His51−Asp75−Ser135) in the active site. NS2B-NS3pro has a somewhat altered specificity relative to other trypsin-like serine proteases. The fact that NS2B-NS3pro recognizes only sites that contain two cationic residues, whereas trypsin recognizes sites containing a single cationic residue, has necessitated the development of

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new classes of inhibitors for targeting the active site.[34] The structural boundaries originally annotated within the polyprotein suggested that the NS3 domain encoded the functional protease. Later studies showed that expression of NS3 alone did not lead to production of an active protease; however, including a portion of NS2B with NS3 leads to full proteolytic activity.[16] The crystal structure of NS2B-NS3pro from dengue virus type 2 shows that the catalytic triad is located between two β-sheets, each formed by six β-strands (Figure 1).

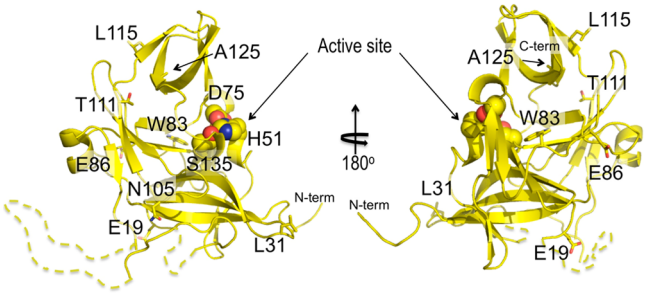

Figure 1. NS2B-NS3pro positions interrogated as potential allosteric sites. Potential allosteric sites were identified on the basis of visual inspection of the known structure of the wild type protease (NS2B-NS3pro) from dengue virus serotype 2 (PDB: 2FOM). The location of residues that were substituted by cysteine are drawn as sticks. The active site triad is drawn as balls. Regions disordered in the crystal structure are drawn as dashed lines. This figure was drawn with PyMOL.

One of the main hurdles in early drug development against this target has been that the NS2B-NS3pro active site is flat, so designing inhibitors by structure-based design has been challenging.[35−37] Another difficulty in finding orthosteric drugs is that NS2B-NS3pro prefers positively charged amino acids in peptide and peptidomimetic substrates. Charged molecules typically display limited oral bioavailability, furthering the difficulty in design of peptidomimetics against NS2B-NS3pro that are effective inhibitors *in vivo*.

The work described here is based on our hypothesis that an alternative functional site, such as an allosteric site, might be identified and targeted for drug development. We define allosteric sites as those regions of the protein that, when bound to a small-molecule ligand, undergo a change in conformation or an alteration in the conformational equilibrium that impacts enzymatic function. Allosteric sites have previously been found to be important in proteases in general (for reviews, see refs 38 and 39), making it likely that allosteric sites in NS2B-NS3pro likewise could be exploited. In this work we have used both novel and known cysteine-reactive chemical probes to identify sensitive regions of NS2B-NS3pro that may serve as functional allosteric sites.

RESULTS AND DISCUSSION

Most studies targeting NS2B-NS3pro by small-molecule inhibitors have focused on the active site, but unfortunately no drug that inhibits the enzyme via active site binding has been approved to date. In this work we reason that finding other sites, potentially allosteric sites that can be targeted for enzyme inhibition, may prove fruitful in drug development against dengue virus. In this study we have interrogated potential allosteric sites in NS2B-NS3pro that can be targeted by small-molecule compounds. Putative allosteric sites spaced across the protein surface were identified by visual inspection of the crystal structure of unliganded NS2B-NS3pro (PDB: 2FOM, Figure 1). Eight individual cysteine residues were introduced near these cavities (Supplementary Figure S1A−D) to provide reactive handles for cysteine-tethering-based covalent-compound binding. Wild-type (WT) NS2B-NS3pro does not have any intrinsic cysteine residues and therefore provides an ideal scaffold for this interrogation. The effects of single cysteine additions on enzyme activity were determined using a GRR-aminomethylcoumarin fluorogenic substrate (Table 1). The majority of the cysteine substitutions had no effect on catalytic activity; however, substitution of cysteine for Trp83, Thr111, or Leu115 fully abolished enzymatic function. Trp83 is a fully buried residue, so it is likely that formation of a cavity and destabilization of the folded form of NS2B-NS3pro results in loss of activity. Thr111 and Leu115 are mostly solvent exposed, so their loss of function is more surprising. Nevertheless, since most of the variants retained catalytic properties similar to the wild-type NS2B-NS3pro, we were able to undertake a cysteine scanning and tethering approach to identify potential allosteric sites.

Our approach to identify new allosteric sites on NS2B-NS3pro shows some similarities to previous versions of cysteine tethering that have led to identification of allosteric sites[40−44] but is unique in that it requires only a small panel of cysteine-reactive probes (Figure 2A). Original tethering libraries intended for ligand discovery were composed of a large number (1,200−40,000) of specific monophores (unique chemical entities or pharmacophores), each attached to a weakly electrophilic thiol moiety. Our panel for allosteric site discovery is composed of monophores that we hypothesized would be broadly reactive and are attached to highly reactive electrophiles. We included the biarylether monophore because in screening of large tethering libraries this monophore was

Table 1. Kinetic Properties of NS2B-NS3pro Variants[a]

variant	K_M (μM)	k_{cat} (min^{-1})	k_{cat}/K_M ($min^{-1}/\mu M$)	molar ratio of DTNB binding	molar ratio of BAClMK binding
wild type	333 ± 21	1.5 ± 0.001	4.5×10^{-3}	0	0
E19C	321 ± 39	1.0 ± 0.10	3.1×10^{-3}	1	1
L31C	343 ± 26	1.0 ± 0.23	2.9×10^{-3}	1	0.8
W83C	ND	ND	–	0.2	NT
E86C	290 ± 51	0.8 ± 0.20	2.7×10^{-3}	1	1
N105C	271 ± 23	1.6 ± 0.03	5.9×10^{-3}	1	1
T111C	ND	ND	–	1	NT
L115C	ND	ND	–	1	NT
A125C	235 ± 45	1.1 ± 0.30	4.6×10^{-3}	1	1

[a]ND = not detectable; NT = not tested.

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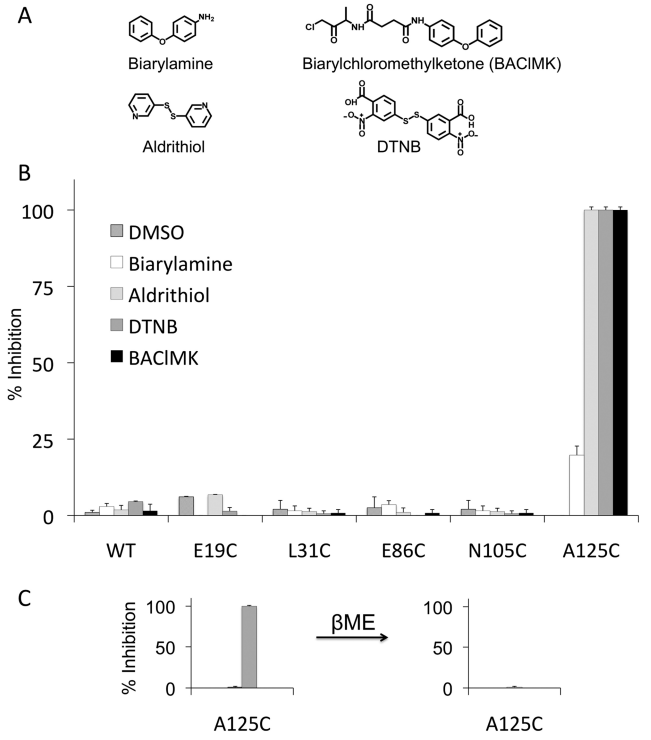

Figure 2. Only A125C is sensitive to chemical probes among the sites tested. (A) Cysteine-reactive probes used in this study. (B) A125C is fully inhibited by all of the cysteine reactive probes including Aldrithiol, DTNB, and biarylchloromethylketone (BAClMK), but not by similar nonreactive probes such as biarylamine. Compound binding at all other sites had no affect on function. (C) The inhibition of A125C by DTNB is relieved by subsequent treatment with the reductant β-mecaptoethanol (β-ME), demonstrating that covalent attachment to the cysteine at A125C is critical for inhibition.

broadly reactive, probably due to its hydrophobic nature and shape, which can be accommodated in many cavities and crevices on protein surfaces. In addition, we included more reactive electrophilic warheads including chloromethylketones and activated disulfides.

Each active cysteine variant was incubated with the panel of cysteine-reactive probes. To monitor the accessibility of the introduced thiols we used DTNB, a quantitative cysteine-reactive colorimetric reagent, which also functions as an inhibitor of A125C NS2B-NS3pro (Figure 2). Most of the cysteine substitutions resulted in shortening of the side chain at the substituted position, with the exception of A125C, which extends the side chain by addition of a thiol. Nevertheless, we found that all of the introduced cysteines were able to bind to DTNB and BAClMK (Table 1), suggesting that all are accessible and reactive. After incubation with the panel of probes, the remaining NS2B-NS3pro activity was measured. All of the introduced cysteines in active versions of NS2B-NS3pro are available, reactive, and fully modified by the probes (Table 1). Two of the inactive variants (T111C and L115C) are fully susceptible to reactions with the introduced cysteine, but W83C only partially reacted. Although the introduced cysteines in the active versions of NS2B-NS3pro are capable of becoming fully modified, we found that only the A125C variant was inhibited by compound binding (Figure 2B). A125C was fully inhibited by DTNB, Aldrithiol, or biarylchloromethylketone (BAClMK, Supplementary Figure S2A), which form covalent adducts to Cys125, indicating that these chemical probes are effective inhibitors at this site. Inhibition by DTNB could be fully reversed by the addition of the reductant (β-mercaptoethanol), indicating that binding was cysteine-dependent (Figure 2C). A125C was only weakly inhibited by the non-cysteine-reactive biarylamine (Figure 2B), suggesting that covalent binding is required for inhibition. A thiol-based biaryl compound (biarylthiol, Supplementary Figure S2B) failed to inhibit A125C under these conditions, suggesting that a more reactive electrophile is required for reaction and inhibition. This also suggests that any interaction between the biaryl monophore and the protein is transient or weak. Covalent modification of A125C by DTNB or BAClMK was readily observed by mass spectrometry (Table 2), but no binding to WT NS2B-NS3pro was observed. This further demonstrates that both DTNB and BAClMK react with the introduced cysteine at A125C but do not react with the active site nucleophile, Ser135, or any other functional group present on the protein. This suggests that NS2B-NS3pro inhibition is not due to a random mechanism and that inhibition is mediated by compound binding specifically to the introduced cysteine at residue position 125.

Table 2. Molecular Weights Observed for NS2B-NS3 Variants Alone or Following Reaction with Cysteine Reactive Compounds[a]

protein	calculated (Da)	observed (Da)
WT	26,296	26,295
WT + BAClMK	26,650	26,294
WT + DTNB	26,494	26,294
A125C	26,329	26,332
A125C + BAClMK	26,682	26,684
A125C + DTNB	26,527	26,526

[a]BAClMK = biarylchlormethylketone, DTNB = 5,5′-dithiobis-(2-nitrobenzoic acid).

To further probe the mechanism of inhibition at A125C, we measured the circular dichroism (CD) spectra to monitor large-scale changes in the structure upon cysteine introduction and compound tethering. Introduction of A125C had no pronounced effect on the CD spectra, suggesting that any changes in the structure of protein were minor (Figure 3). This finding is also consistent with the observation that the catalytic properties of A125C were essentially unchanged from WT. Binding of DTNB also had no noticeable effect on the CD

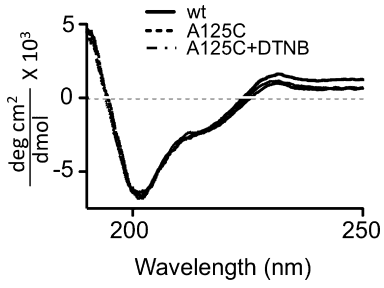

Figure 3. A125C NS2B-NS3pro achieves the same global structure as wild-type before or after DTNB binding. The nearly superimposable circular dichroism (CD) spectra for NS2B-NS3pro wild-type and A125C in the presence or absence of the inhibitor DTNB suggests that neither cysteine substitution nor compound binding have any large-scale or disruptive effects on the structure of the protein.

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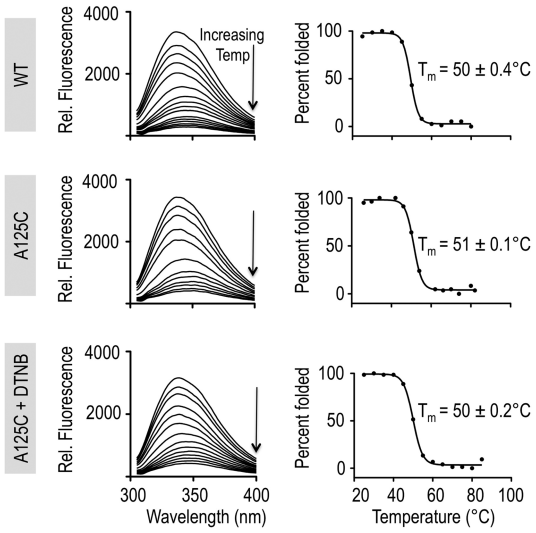
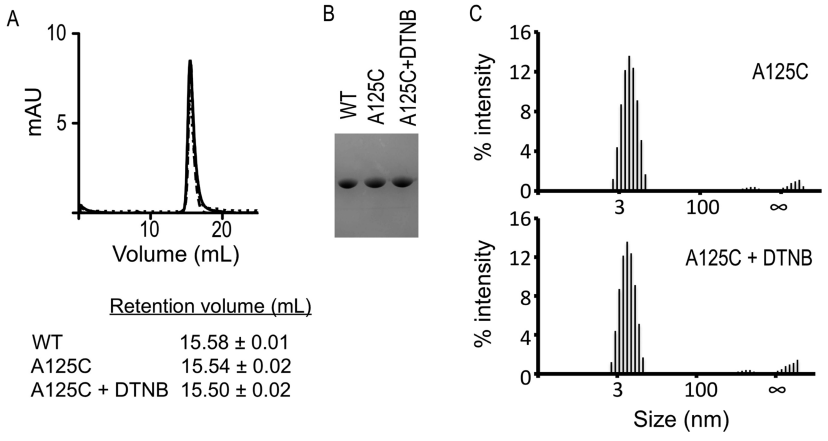


Figure 4. Inhibitor binding does not affect A125C thermal stability. The thermal stability as assessed by intrinsic tryptophan fluorescence of NS2B-NS3pro wild-type and A125C in the presence or absence of the inhibitor DTNB are similar, suggesting that neither cysteine substitution nor compound binding have any significant effect on stability or on the folded/unfolded equilibrium for the protein.

spectra, suggesting that the mechanism of DTNB-based inhibition was not through unfolding or disruption of the global NS2B-NS3pro structure.

Given that DTNB binding did not impact the overall structure, we explored whether compound binding would impact the stability of the protein. Thermal denaturation of NS2B-NS3pro as monitored by circular dichroism showed that WT, A125C, and DTNB-bound A125C all had T_m's of 50 ± 1 °C, suggesting that DTNB binding has no effect on stability. In addition, we monitored the impact of DTNB binding on other cysteine insertion variants. E19C, L31C, E86C, and N105C versions of NS2B-NS3pro all also showed T_ms similar to those of WT and A125C and changes in T_m of 1 °C or less upon binding of DTNB (Supplementary Figure S3), suggesting that the mode of interaction is similar between all of the variants. Thermal denaturation of NS2B-NS3pro results in an unusual, but not unprecedented,[45] gain in CD signal; therefore, we also monitored stability using intrinsic tryptophan fluorescence. A125C showed stability indistinguishable from WT and DTNB binding also had no impact on stability (Figure 4). The above findings suggest that the structures of A125C and the A125C complexes with ligands are similar to WT NS2B-NS3pro and that covalent compound binding resulting in the inhibited form is not altering the folding-unfolding equilibrium.

One of the most common sources of false positives in early drug discovery is formation of organic small-molecule colloidal aggregates or small molecule-protein co-aggregates, which can sometimes inhibit enzymes nonspecifically.[46] The reasons for these phenomena are not well understood, but a possible mechanism is that single enzyme molecules bind tightly and nonspecifically to small compound aggregates, thereby leading to enzyme inactivation in protein:small-molecule co-aggregates. This kind of inhibition can be problematic, particularly with hydrophobic compounds such as the probes used here, and in our case could lead to spurious identification of allosteric sites. To investigate whether this type of mechanism is involved in inhibition of A125C, we subjected A125C to size exclusion chromatography, native gel electrophoresis, and dynamic light scattering (Figure 5). WT, A125C, and DTNB-bound A125C all showed identical retention on a size-exclusion column, which was consistent with a monomer of 26 kDa (Figure 5A). Likewise, WT, A125C, and DTNB-bound A125C all migrated at the same position on a native gel, suggesting that all three species are monomers of similar mobility (Figure 5B). This also indicates that no intermolecular disulfides are forming that could be responsible for the observed loss of activity for DTNB-bound A125C. Finally, A125C had a comparable distribution of particle sizes by dynamic light scattering, further suggesting that the inhibited form of A125C is fully monomeric, soluble, and well behaved, even though it is fully inhibited (Figure 5C). These analyses all suggest that no spurious or aggregation-mediated mechanisms of inhibition are



Retention volume (mL)

WT	15.58 ± 0.01
A125C	15.54 ± 0.02
A125C + DTNB	15.50 ± 0.02

Figure 5. A125C is monomeric even in the presence of covalent inhibitors. (A) The oligomeric states of wild-type and A125C NS2B-NS3pro in the presence or absence of the inhibitor DTNB were investigated by size exclusion chromatography. The similar retention times and elution profiles suggest that neither the introduced cysteine nor DTNB have any impact on oligomeric state, which is consistent with a monomer of 26 kDa. (B) Wild-type and A125C NS2B-NS3pro in the presence or absence of the inhibitor DTNB all display similar migration patterns in native gel electrophoresis, indicating that neither cysteine substitution nor DTNB change the oligomeric state. (C) A125C NS2B-NS3pro in the presence or absence of the inhibitor DTNB show similar profiles by dynamic light scattering, suggesting that DTNB does not influence the aggregation state of the protein.

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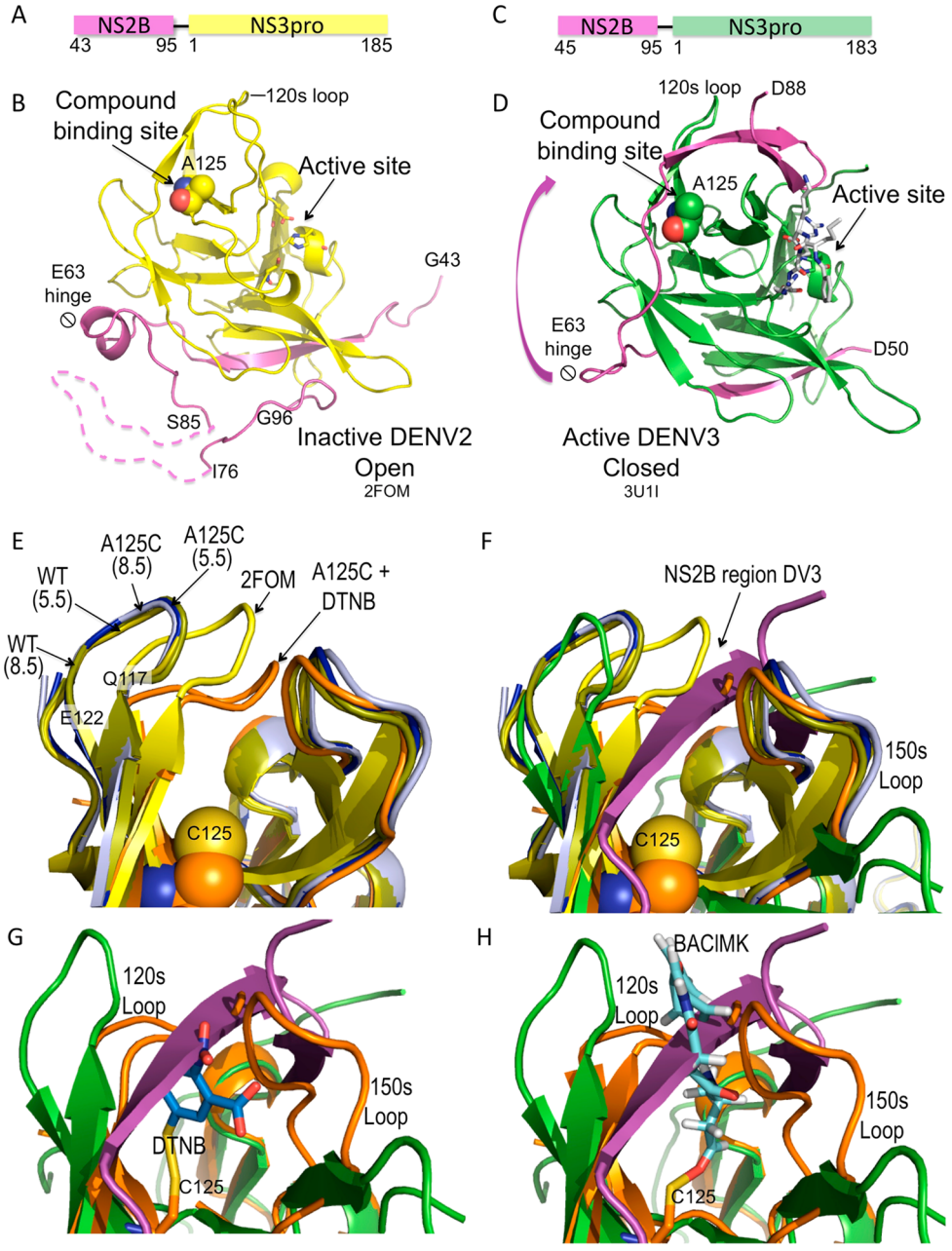

Figure 6. The 120s loop is flexible and undergoes conformational changes in the presence of DTNB. (A) Domain architecture and numbering of NS2B-NS3pro from dengue virus serotype 2 (DENV2). (B) The structure of unliganded (open, inactive) NS2B-NS3pro from DENV2 (yellow and purple ribbons, PDB: 2FOM). Ala125 is drawn as spheres; the catalytic triad is drawn as sticks. (C) Domain architecture and numbering of NS2B-NS3pro from dengue virus serotype 3 (DENV3), which is 68% identical to DENV2 NS2B-NS3pro. (D) The structure of DENV3 NS2B-NS3pro (green and purple ribbons, PDB: 3U1I) bound to the substrate-like, active-site inhibitor benzoly-Nle-Lys-Arg-Arg-aldehyde (white sticks) shows the active conformation of NS2B-NS3pro. Upon substrate binding the NS2B region (purple) from residues 63 to 88 moves around the hinge at residue 63 to interact with the Ala125-adjacent region between the 120s and 150s loops. A125C is drawn as spheres; the catalytic triad is drawn as sticks. (E) The 120s loop shows conformational flexibility. The structures of NS2B-NS3pro WT at pH 5.5 (olive) or 8.5 (dark olive) and A125C at pH 5.5 (dark blue) or 8.5 (light blue) all show the same conformation of the 120s loop. In contrast, in the presence of DTNB the 120s loop moves to a new (down) conformation (orange). The previously reported WT structure at pH 5.5 (PDB: 2FOM, yellow) shows an intermediate conformation of the 120s loop indicating that this loop is flexible and able to adopt a range of conformations. (F) The conformation of the 120s loop in the presence of DTNB (orange) sterically clashes with the substrate-bound conformation that has been observed in DENV3 NS2B-NS3pro (purple and green). Residues 73−88 from the NS2B region (purple) move from the open position in the absence of substrate to the closed position, which is incompatible with the conformation observed in the presence of DTNB. (G) Computational model of DTNB bound to A125C showing steric clash between DTNB and the NS2B region of the active form of DENV3 NS2B-NS3pro (green and purple strands). (H) Computational model of BAClMK bound to A125C showing steric clash between BAClMK and the NS2B region (purple strands). These structures were drawn with PyMOL.

responsible for the loss of activity conferred by DTNB and the other A125C inhibitors.

Together, these results suggest that targeting the 120s loop region, or potentially a cavity or crevice near the Ala125 site,

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with a small molecule inhibitor may be an effective way to achieve enzyme inhibition. To visualize the mechanisms of DTNB and BAClMK inhibition of NS2B-NS3pro, we sought to determine the crystal structures of A125C in complex with DTNB or BAClMK. We first determined the structures of unliganded WT and A125C (Supplementary Table S1) under pH 5.5 conditions, similar to those observed previously for WT NS2B-NS3pro from dengue virus serotype 2 (DENV2[35]). Both WT and A125C had the same overall fold as the published structure of WT NS2B-NS3pro (PDB: 2FOM[35]) except that the 120s loop (residues 117−122, which are ∼15 Å away from the substrate-binding groove) was in a different conformation. Because two of the covalent inhibitors, BAClMK and DTNB, do not react to form a covalent adduct with A125C at pH 5.5 but do bind and robustly inhibit NS2B-NS3pro at pH 8.5, we sought alternative, neutral pH crystallization conditions. Extensive screening of unliganded NS2B-NS3pro failed to produce any new crystallization conditions. Numerous screens of NS2B-NS3pro covalently modified with BAClMK or DTNB also failed to produce crystals under any conditions, so we developed a method for transferring the unliganded A125C pH 5.5 crystals to pH 8.5 without negatively impacting diffraction (Supplementary Table S1). Crystals subsequently soaked with BAClMK failed to diffract (Supplementary Table S1). Fortunately, we were able to soak DTNB into NS2B-NS3pro crystals, yielding viable crystals that diffracted to 1.74 Å resolution (Supplementary Table S1). Analysis of this structure in detail suggested that conformational changes in the 120s loop could be important.

In the absence of substrate, DENV2 NS2B-NS3pro can adopt what is termed the open (inactive) conformation, wherein the NS2B region (Figure 6A) sits at the base of the protein below the active site (Figure 6B). No crystal structures of NS2B-NS3pro from DENV2 have been reported with substrate or active-site inhibitors bound; however, the molecular details of the closed (active) conformation are available based on homology to the structure of NS2B-NS3pro from dengue virus serotype 3 (DENV3), which is 68% identical to that of DENV2 (Figure 6C). A structure of the closed form of DENV3 NS2B-NS3pro was determined in the presence of a substrate-like active-site inhibitor (Nle-Gly-Lys-Lys-aldehyde). In the presence of active-site inhibitor, the NS2B region undergoes a large conformational rearrangement such that residues 73−88 sit above the active site near the 120s loop (Figure 6D). It appears that this conformational rearrangement is required for activity and is essential for substrate binding and catalytic turn over. Recent extensive NMR analysis suggests that in solution NS2B-NS3pro favors the closed conformation in the presence and absence of substrate.[47] Thus, flexibility of the NS2B region of the protease is critical for function.

The 120s loop also appears to be quite flexible; we and others have observed a number of conformations of this loop in unliganded versions of WT and A125C NS2B-NS3pro (Figure 6E). The conformation of the 120s loop in the DTNB-soaked structure is incompatible with the active (closed) conformation of the NS2B region. Superposition of the DTNB-soaked structure with the active conformation of NS2B-NS3pro from DENV3 shows a steric clash between the 120s loop and the active conformation of NS2B (Figure 6F). While we were not able to observe any density for DTNB attached to the cysteine residue at position 125 (Cys125), we are certain that the reaction between Cys125 and DTNB occurred in the crystals due to the appearance of a yellow color resulting from the DTNB reaction. In contrast, the same reaction did not occur with mother liquor alone. The presence of large (30 Å diameter) solvent channels in the NS2B-NS3pro crystals (Supplementary Figure S4) appears to provide ample access of DTNB to the A125C residues throughout the crystals. To thoroughly explore the possibility of weak density or multiple conformations for DTNB, we used placement based on Glide[48,49] with density-guided and force-field-based refinement. While the overall model statistics improve with the PrimeX refinement (Supplementary Table S1), no models could be generated that place DTNB in areas of significant electron density. As such, we hypothesize that DTNB may have incompletely reacted, dissociated after freezing or during synchrotron radiation, or is dramatically disordered.

Although we were unable to observe any electron density for DTNB attached to the cysteine at position 125 (Supplementary Figure S5), the presence of DTNB in the crystal does have a notable effect on the structure of A125C we observe. In addition to the altered conformation of the 120s loop, residues Thr77−Met84 are ordered in the DTNB-soaked structure; these residues were disordered in all structures to date of DENV2 NS2B-NS3pro including the high-resolution structures 2FOM (1.5 Å) and the WT pH 5.5 structure reported here (1.46 Å). We assume that the NS2B region of NS2B-NS3pro is in equilibrium between the closed (active) and open (inactive) conformations; it appears that the presence of DTNB may stabilize the open (inactive) conformation. Only in the presence of DTNB can we observe density for the Thr77−Met88 stretch, which is ordered by crystal contacts between Thr76 and Asn119 in the 120s loop (Supplementary Figure S6). Thus, one model of DTNB-based inhibition is that this steric clash between the flexible 120s loop and the active conformation of the NS2B region is responsible for the inhibitory activity of DTNB.

A second model of inhibition by DTNB and BAClMK also involves NS2B reorganization. Modeling covalent binding of either DTNB (Figure 6G) or BAClMK (Figure 6H) to C125, we see that a likely conformation for the inhibitor is protruding toward the 120s loop in a position that would sterically clash with the active conformation of the NS2B region. This steric clash would prevent the NS2B region from adopting the closed (active) conformation, thereby shifting the ensemble toward the open (inactive) conformation we observe in the DTNB-soaked structure. We favor this model for explaining the inhibition of NS2B-NS3pro activity with three structurally divergent compounds, all of which could utilize the same mechanism. Due to the covalent nature of the interactions between the probes and NS2B-NS3pro variants, we expect that if any other interrogated sites were equally susceptible to allosteric inhibition, our approach would have also recognized those sites. Given that only A125C was inhibited by any of the compounds, we posit that this region is one of the most susceptible regions of the protein for allosteric inhibition. We predict that small molecules that bind between the 120s loop and the 150s loop (residues 153−164, which sit below the S3 subsite) will serve as effective inhibitors of NS2B-NS3 protease activity (Supplementary Figure S7).

Conclusions. The approach we developed in this work allowed us to identify the Ala125 allosteric site in NS2B-NS3pro and is promising for future applications due to its simplicity and feasibility. In this study, interrogating just eight single-site cysteine substitutions with a panel of just five chemical probes, three of which are commercially available, we

were able to identify a productive allosteric site that was susceptible to full inhibition by three diverse molecules. The approach we describe is also generally applicable for allosteric site identification in a wide range of protein targets. This cysteine-based approach is particularly well suited to target proteins such as NS2B-NS3pro that lack native cysteines. The lack of cysteines need not be a requirement for success, provided that at least some of the probes have no effect on the function of the native target protein, or as is often the case, removal of reactive surface cysteines has no impact on function.

Prior to this study several pieces of evidence have suggested that the dengue virus protease family should be susceptible to allosteric inhibition. The 3F10 antibody disrupts the interaction of NS2B with NS3 *in vitro*, suggesting that allosteric inhibition of NS2B-NS3pro should be possible.[50] Antibody-based inhibition of proteases has been used for half a century, and some of inhibitory antibodies utilize allosteric mechanisms (for a review, see ref 51). For example, another trypsin-like protease, hepatocyte growth-factor activator, can be allosterically inhibited by the Fab75 antibody through binding an active-site adjacent loop (residues 99−102).[52] Although the homologous region is a short turn in NS2B-NS3pro (residues 72−74) and thus not useful as an allosteric epitope, we can envision that a similar inhibitory mechanism could be used by an NS2B-NS3pro 120s- or 150s-specific antibody. Finally, compounds derived from *in silico* docking experiments that function as noncompetitive inhibitors have also been reported.[53] While we are unable to ascertain whether an innate biological role of the Ala125 allosteric site exists, it is our aim to continue to assess the native role and exploit this site chemically with specific, noncovalent ligands or antibodies.

The prevalence of diseases caused by flaviviruses is rapidly expanding, raising interest in the proteases across this family as drug targets. A similar conformational change in the NS2B region is observed for the protease from West Nile virus, which shares 46.8% identity with DENV2 NS2B-NS3pro, suggesting that the mechanism of activation may be conserved across the family. In the unliganded state, the NS2B region of NS2B-NS3pro from West Nile virus is in the open conformation. When substrate binds, the NS2B region moves above the 120s loop (Supplementary Figure S7), adopting the closed conformation. Given the similarity in the mechanism of activation, we predict that the protease from West Nile virus would also be allosterically inhibited in a similar manner at the region of the 120s and 150s loops. In fact, uncompetitive/allosteric inhibitors of West Nile virus have been previously described, but no structural information on the site of binding has been reported. It is tempting to consider whether the Ala125 region could be the target of the pyrazole-ester-based West Nile NS2B-NS3 protease inhibitors.[54,55]

Allosteric inhibition of dengue virus protease is of potential interest due to the lack of clinically available inhibitors that target the NS2B-NS3pro active site. All small molecule binding sites, whether they be allosteric or otherwise, are composed of three components: a cavity (which may be induced by a small molecule), polar residues that contribute directional forces allowing specificity, and hydrophobic interactions that provide the enthalpy for binding. The region identified near Ala125 appears to possess these three essential elements. Ala125 sits between the 120s and 150s loops, which our work has shown to be very flexible. Thus it appears that the shape of this cavity is malleable, potentially accommodating a number of chemical entities in addition to the BAClMK and DTNB. This region also has a number of appropriately positioned polar residues (e.g., Asn119, Thr118, and Thr156) for providing specificity and hydrophobic residues (e.g., Phe116, Ile123, Val154, Val155, and Val162) for providing binding affinity (enthalpy). Our studies use a covalently linked version of NS2B-NS3pro that has been widely utilized and characterized.[35] Recently Keller and co-workers have shown that unlinked NS2B-NS3pro exhibits slightly higher activity (up to 5-fold) and appears to prefer the closed conformation even prior to substrate binding.[56] Nevertheless, we anticipate that the allosteric site identified at Ala125 will also be effective against the unlinked NS2B-NS3pro, but that remains to be tested. Development of noncovalent inhibitors that utilize the site we have identified is not trivial, but prior work has demonstrated that covalent tethering hits can effectively be developed into noncovalent inhibitors.[40,57,58] Thus we envision that both structure-guided rational approaches that take loop flexibility into account and empirical screening for inhibitors against NS2B-NS3pro may be fruitful routes to further exploitation of this allosteric site.

Drug resistance becomes an issue in any rapidly evolving system, including pathogenic human viruses such as dengue virus, so the development of allosteric inhibitors requires careful consideration. While combination treatments affecting two protein targets have been widely used in viral therapy, the combination of active-site and allosteric inhibitors against a single protein target has not been tested. We hypothesize that simultaneously targeting both the active and allosteric sites of NS2B-NS3pro should delay the onset of resistance over single-site inhibition. At the very least, the system described here will enable us to measure development of resistance in single-target combination therapy and directly assess whether active- or allosteric-site inhibitors are more prone to development of resistance in NS2B-NS3pro. In the best-case scenario, one might envision development of clinically useful allosteric inhibitors that target the allosteric site we have identified.

METHODS

Methods for all aspects of this work are contained in the Supporting Information.

ASSOCIATED CONTENT

Supporting Information

This material is available free of charge via the Internet at http://pubs.acs.org.

Accession Codes

The structures described here have been deposited in the Protein Data Bank with assession codes 4M9F, 4M9I, 4M9K, 4M9M, and 4M9T.

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The authors declare no competing financial interest.

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■ ABBREVIATIONS

BAClMK, biarylchloromethylketone; DTNB, 5,5′-dithiobis-(2-nitrobenzoic acid); DENV, dengue virus; DCM, dichloromethane

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