Biochemistry

Article

Cite This: Biochemistry XXXX, XXX, XXX−XXX

pubs.acs.org/biochemistry

Cysteine Disulfide Traps Reveal Distinct Conformational Ensembles in Dengue Virus NS2B-NS3 Protease

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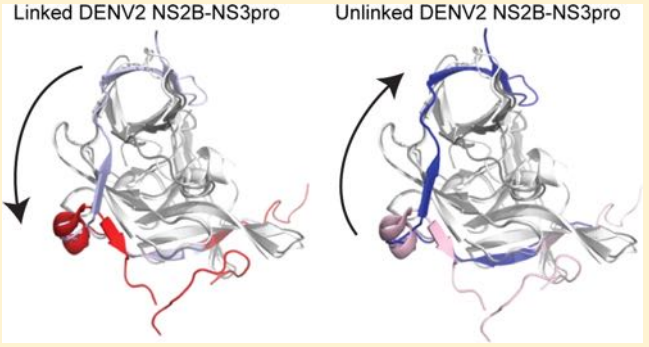
Supporting Information

ABSTRACT: The dengue virus protease (NS2B-NS3pro) plays a critical role in the dengue viral life cycle, making it an attractive drug target for dengue-related pathologies, including dengue hemorrhagic fever. A number of studies indicate that NS2B-NS3pro undergoes a transition between two widely different conformational states: an "open" (inactive) conformation and a "closed" (active) conformation. For the past several years, the equilibrium between these states and the resting conformation of NS2B-NS3pro have been debated, although a strong consensus is emerging. To investigate the importance of such conformational states, we developed versions of NS2B-NS3pro that allow us to trap the enzyme in various distinct conformations. Our data from these variants suggest that the enzymatic activity appears to be dependent on the movement of NS2B and may rely on the flexibility of the protease core. Locking the enzyme into the "closed" conformation dramatically increased activity, strongly suggesting that the "closed" conformation is the active conformation. The observed resting state of the enzyme depends largely on the construct used to express the NS2B-NS3pro complex. In an "unlinked" construct, in which the NS2B and NS3 regions exist as independent, co-expressed polypeptides, the enzyme rests predominantly in a "closed", active conformation. In contrast, in a "linked" construct, in which NS2B and NS3 are attached by a nine-amino acid linker, NS2B-NS3pro adopts a more relaxed, alternative conformation. Nevertheless, even the unlinked construct samples both the "closed" and other alternative conformations. Given our findings, and the more realistic resemblance of NS2B-NS3pro to the native enzyme, these data strongly suggest that studies should focus on the "unlinked" constructs moving forward. Additionally, the results from these studies provide a more detailed understanding of the various poses of the dengue virus NS2B-NS3 protease and should help guide future drug discovery efforts aimed at this enzyme.



Dengue fever, dengue hemorrhagic disease, and dengue shock syndrome are mosquito-borne diseases caused by dengue virus infection. Dengue virus is a member of the flavivirus family, which consists of more than 70 different viruses, including Zika virus, West Nile virus, and yellow fever virus.[1] The four antigenically distinct dengue virus serotypes (DENV1−DENV4) are >65% genetically homologous.[2] Infection with any of these individual serotypes can result in full medical manifestation of dengue-related maladies. These diseases remain a global health threat for the 3.9 billion people living in infected tropical regions[3] that result in an estimated 390 million infections annually.[4] In 2015, a DENV vaccine (Dengvaxia) was approved in Mexico, the Philippines, and Brazil. However, the vaccine lacks consistent efficacy against each serotype[5] and is recommended only for individuals that have been previously exposed to dengue virus,[6] leaving an enormous population still at risk. At present, no approved antiviral treatments are available to treat either dengue virus infections or related pathologies.

The linear, positive-sense RNA genome of dengue virus is translated into a single-chain polyprotein precursor that consists of three structural proteins that form the viral coat [capsid (C), membrane (M), and envelope (E)] and five nonstructural proteins (NS1−NS5 arranged as C-prM-E-NS1-NS2A-NS2B-NS3-NS4A-NS4B-NS5). Maturation of the precursor is one of the key steps in the viral life cycle, which requires proteolytic processing by the dengue virus protease (NS2B-NS3pro) to release individual viral proteins from the intact polyprotein chain.[7,8] NS2B-NS3pro cleaves the viral protein at the NS2A/NS2B, NS2B/NS3, NS3/NS4A, and NS4B/NS5 junctions as well as within the capsid protein.[9,10] Full processing releases the viral protease (NS2B-NS3pro), helicase (NS3),[11] methyltransferase (NS5), and RNA-dependent RNA polymerase (NS5).[12]

Because of its critical role in polyprotein maturation and viral infectivity, NS2B-NS3pro has been studied intensively. Structural studies of NS2B-NS3pro from DENV1,[13] DENV2,[14] DENV3,[15] West Nile virus (WNV),[14] and Zika virus (ZIKV)[16]

Received: September 11, 2018
Revised: November 21, 2018
Published: November 26, 2018

DOI: 10.1021/acs.biochem.8b00978
Biochemistry XXXX, XXX, XXX−XXX

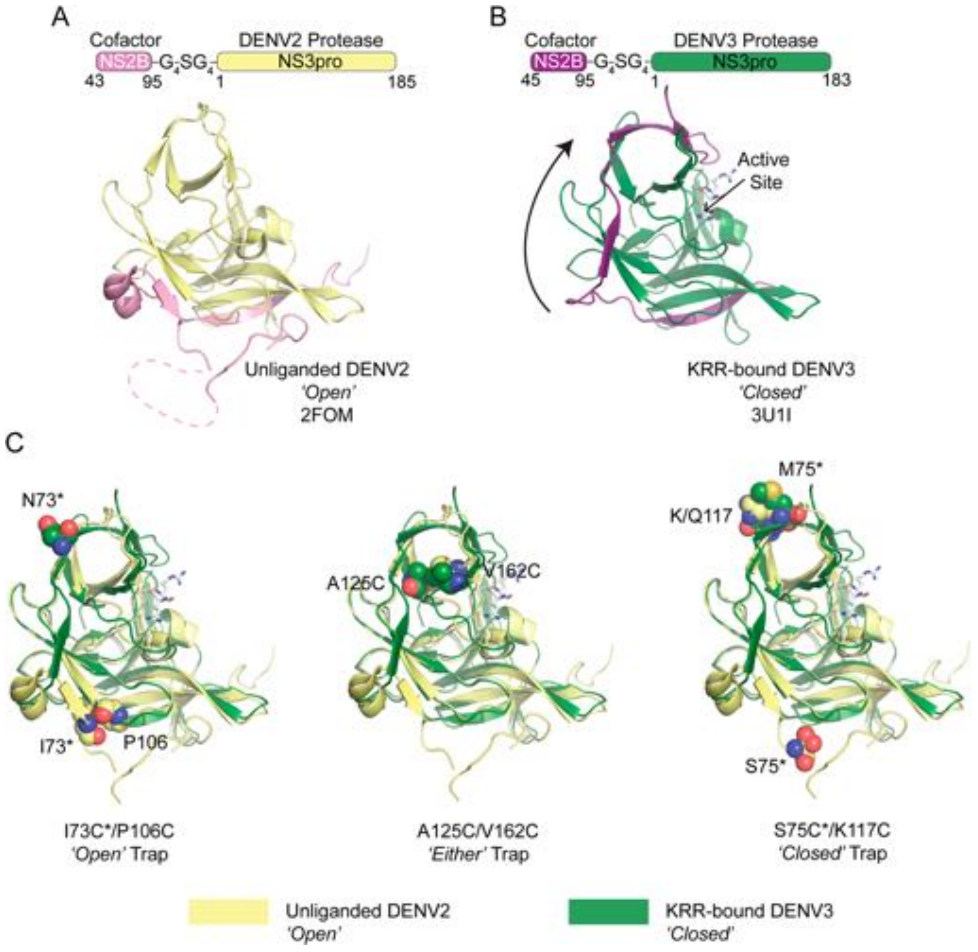


Figure 1. Conformational flexibility of DENV NS2B-NS3pro inspired cysteine trap designs. (A) DENV2 NS2B-NS3pro in the "open" conformation (PDB entry 2FOM), with the NS2B cofactor colored pink and the NS3 protease domain colored yellow. (B) DENV3 NS2B-NS3pro in the "closed" conformation (PDB entry 3U1I), with the NS2B cofactor colored purple and the NS3 protease domain colored green. (C) Cysteine trap variants were designed to capture the "open" conformation with the I73C*/P106C pair, the "either" conformation with the A125C/V162C pair, or the "closed" conformation with the S75C*/K117C pair. Each cysteine pair (spheres) designed to form a trap is drawn on DENV2 "open" (yellow) or DENV3 "closed" (green).

indicate that NS2B-NS3pro shares similar structural features across the closely related flavivirus genus. While the protease was originally believed to be encoded by NS3, early structural and biochemical studies showed that the protease is a two-component system such that a portion of NS2B in addition to the protease portion of NS3 is also required for proteolytic activity.[7] Multiple types of protein constructs have been used for NS2B-NS3pro expression. In one type, a $Gly_4SerGly_4$ linker joins NS2B to NS3pro,[17] which was reported to improve expression and purification, while another type requires co-expression of the unlinked NS2B and NS3 regions.[18,19] The catalytic activity is slightly different between the linked and unlinked protein constructs, with the unlinked form showing activity approximately 0−5-fold greater than that of the linked form,[18] likely due to constraints introduced by the synthetic linker. On the other hand, there is some controversy about the flexible nature of NS2B-NS3pro, particularly of that expressed from linked and unlinked protein constructs.

Crystallographic studies indicate that the C-terminal portion of the NS2B cofactor undergoes a large conformation change in the presence of an inhibitor (Figure 1A,B). High-resolution crystal structures of linked NS2B-NS3pro without a substrate from DENV2,[14] DENV3,[15] and WNV[20] show NS2B in the "open" conformation (Figure 1A). Interestingly, linked apo NS2B-NS3pro from ZIKV has been crystallized in a variety conformations, including a "pre-open" state[21] and a "relaxed" state.[22] Linked NS2B-NS3pro consistently adopts the "closed" conformation upon substrate binding (Figure 1B) as was seen in the DENV3,[15] WNV,[14] and ZVP[16] structures; however, a structure of substrate-bound DENV2 NS3B-NS3pro has not yet been attained. Thus, there is a lack of data regarding the conformation of the NS2B region for DENV2 NS2B-NS3pro crystallographically.

The assortment of states observed for the NS2B C-terminal portion suggests that this region is highly mobile. To study the flexibility of NS2B, several nuclear magnetic resonance (NMR) studies have been performed on the NS2B-NS3pro constructs from DENV, WNV, and ZIKV. In the presence of inhibitors, the C-terminal region of NS2B adopts the "closed" conformation,[23−27] in addition to the "closed" conformation being observed in a linked WNV construct without an inhibitor.[23] In DENV constructs lacking the synthetic $Gly_4SerGly_4$ linker, NMR spectroscopy further suggested a "closed" conformation.[28,29] In summary, most NMR findings suggested that NS2B-NS3pro rests in the "closed", active state regardless of the presence of an artificial linker joining the cofactor to the

DOI: 10.1021/acs.biochem.8b00978
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protease domain or the presence of an inhibitor. However, NMR studies also indicate that NS2B was flexible and that inhibitors bound to the active site stabilized the "closed" state.[24,25,28,30] Together, these data suggested that the C-terminal portion of NS2B may adopt numerous conformations, despite recent suggestions that the only relevant conformation for drug development is the "closed" conformation.[31] Such flexibility may offer unique opportunities for drug development to inactivate the protease and thus prevent viral replication. Although NMR and X-ray studies suggest different equilibria between the "closed" and "open" states of NS2B in unlinked and linked forms of NS2B-NS3pro, data from both techniques agree about the flexible nature of NS2B. Unfortunately, data comparing the linked and unlinked constructs are often overgeneralized by equating results between different proteases under different conditions, confounding the interpretation.

From a drug discovery standpoint, the controversy over the relevance and relative population of the "open" and "closed" conformations of the NS2B-NS3pro flaviviral proteases is significant as the conformational equilibrium defines the molecular target. Here we compared the ability of the linked and unlinked constructs to adopt these different poses side by side and found that the unlinked construct rests predominantly in the "closed" conformation while the linked construct adopts an alternative conformation. Although we and others have used the linked construct in the past, these studies suggest that the unlinked construct should be used exclusively going forward. Nonetheless, the unlinked enzyme retains the ability to adopt the "open" state, which we verify is inactivating. Our data also suggest that the flexibility of the core of the enzyme may be important for NS2B-NS3pro catalytic activity, perhaps allowing another route for inhibition.

METHODS

Dengue Virus Serotype 2 Protease (DENV2 NS2B-NS3pro) Expression Constructs. The wild-type dengue virus serotype 2 protease (NS2B-NS3pro) linked construct encoding a synthetic gene (Celtek Bioscience) for *Escherichia coli* codon-optimized N-terminally His_6-tagged dengue virus serotype 2 protease (DENV2 NS2B-NS3pro) gene was constructed by ligation into the XhoI/BamHI sites of the pET15b vector (Strategene). This construct expresses a protein comprising amino acids (aa) His_6-NS2B (aa 45−95)-GGGGS-GGGG-NS3pro (aa 1−185). The wild-type DENV2 unlinked construct, a gift from T. Keller,[18] encodes NS2B (aa 48−100) in the pACYDUET vector and NS3pro (aa 1−187) in the pETDUET vector. Amino acid substitutions were introduced by QuikChange site-directed mutagenesis in the construct encoding NS2B (aa 48−95) in the pACYDuet vector and NS3pro (aa 1−185) in the pETDUET vector to better match the linked construct lengths. All DNA sequences were validated by DNA sequencing (Genewiz).

WT and Cysteine Variant Expression and Purification. NS2B-NS3pro expression constructs were transformed into the BL21 (DE3) T7 Express strain of *E. coli* (New England Biolabs). Cells transformed with the linked constructs were grown in 2×YT medium with ampicillin (100 μg/L), and unlinked constructs were grown in 2×YT medium with chloramphenicol (34 μg/L) and ampicillin (100 μg/mL) at 37 °C until they reached an optical density at 600 nm of 0.8. The temperature was decreased to 25 °C, and cells were induced with 1 mM isopropyl β-D-1-thiogalactopyranoside to express protein. Cells were harvested after expression for 3 h and pelleted by centrifugation at 4700*g* in an SLC4000 rotor (Sorvall). Cell pellets were stored at −80 °C, frozen and thawed, and lysed by microfluidization (Microfluidics, Inc.). At no point during the purification process were reductants included in the buffers, nor were the proteins exposed to any added reducing agents. For microfluidization, cells were resuspended in a buffer containing 50 mM Tris (pH 8.5), 50 mM NaCl, 5% glycerol, and 2 mM imidazole (lysis buffer). Lysed cells were centrifuged at 27000*g* for 1 h to remove cellular debris. The supernatant was loaded onto a 5 mL HiTrap Ni-affinity column (GE Healthcare) equilibrated with lysis buffer. The column was washed with a buffer consisting of 50 mM Tris (pH 8.5), 50 mM NaCl, 5% glycerol, and 10 mM imidazole, and the protein was eluted with the buffer containing 50 mM Tris (pH 8.5), 50 mM NaCl, 5% glycerol, and 300 mM imidazole. Tris buffers were prepared by mixing the appropriate ratio of Tris base and Tris-HCl to achieve the desired pH.

In the unlinked construct, the His_6 tag is on the NS3 domain. Thus, in the unlinked version, any uncomplexed NS2B would not be isolated by our strategy. The NS3 domain is insoluble, but we did not observe any insoluble protein, suggesting that all of the NS3 was in complex with NS2B, which enables the formation of the fully folded soluble protease. The eluted fractions were diluted 6-fold with 50 mM Tris buffer (pH 8.5), to decrease the salt concentration. This protein sample was purified further by anion exchange chromatography. The partially purified sample was loaded onto a 5 mL HP High Q column (GE Healthcare) and eluted with a linear NaCl gradient. NS2B-NS3pro eluted in a buffer consisting of 50 mM Tris (pH 8.5) and 150 mM NaCl.

For the linked and unlinked constructs, no reductant was present during any steps of the purification. Purification typically occurs over the course of 5−6 h. During the entire purification, the protein is stored on ice or subjected to chromatography in a refrigerated cabinet where all instruments are cooled to 4 °C. Prior to the assays, proteins were thawed on ice and incubated for approximately 30−60 min on ice during analysis.

Quantification of Free Thiols. To monitor the level of disulfide formation, free thiols in NS2B-NS3pro samples were measured using the quantitative, colorimetric, thiol-reactive reagent DTNB. The purity of NS2B-NS3pro was estimated from a Coomassie-stained sodium dodecyl sulfate−polyacrylamide gel electrophoresis (SDS−PAGE) gel. The purity estimates ranged from 80 to 98%. The concentration of NS2B-NS3pro was calculated by multiplying the purity factor by the concentration calculated from A_{280} with an ε of 41940 M^{-1} cm^{-1}. A standard curve for DTNB absorbance was prepared using DTT samples at concentrations from 0 to 30 μM; 240 μL of 10 μM NS2B-NS3pro double cysteine substitution variants was added to 10 μL of 1 M Tris (pH 8.0) and 40 μL of 50 mg/mL (saturated) DTNB diluted in H_2O with insoluble DTNB allowed to settle. The absorbance of each sample was monitored at 420 nm, and the free thiol concentration was read from the DTT standard curve. Because DTT reacts with DTNB and because removal of DTT immediately allows return from the fully reduced form to the native disulfide equilibrium, it was impossible to perform an assessment of the fully reduced state of NS2B-NS3pro variants unless DTT was present. Unfortunately, all the reductants that were tested, DTT, β-mercaptoethanol, and TCEP [tris(2-carboxyethyl)phosphine], reacted strongly with DTNB, thus preventing accurate assessment of disulfide bond formation by DTNB in the fully reduced state.

DOI: 10.1021/acs.biochem.8b00978
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To obtain reproducible results, it was essential to use the DTT standard curve and perform relevant comparisons on the same day. The data shown are the averages of three independent experiments performed on three separate days. To visualize the disulfide-bonded samples, 5 μM protein was separated on an SDS−PAGE nonreducing gel in the presence or absence of DTT.

Differential Scanning Fluorimetry (DSF). Protein (15 μM) was incubated with and without 2 mM DTT for 30 min at room temperature in 25 mM HEPES (pH 7.5) and 75 mM NaCl. The reaction solution was mixed with a final concentration of 5× SYPRO Orange dye before stability was measured in a CFX Connect RT-PCR detection system (Bio-Rad) using a final 50 μL reaction volume in duplicate. The fluorescence was measured at 0.5 °C intervals over the range of 25−95 °C. The thermal melting temperature (T_m) was calculated by fitting the curves to buffer-subtracted values using Prism (GraphPad Software).

4-(2-Aminoethyl)benzenesulfonyl Fluoride Hydrochloride (AEBSF) Induction of the "Closed" Conformation. AEBSF has previously been shown to inhibit DENV2 NS2B-NS3pro in a dose-dependent manner. In prior reports, even after incubation for only 10 min, ∼20% inhibition was observed at 500 μM AEBSF. To achieve greater inhibition, we used a longer incubation and a higher AEBSF concentration. The linked "closed" (S75C*/K117C) and "open" (I73C*/P106C) constructs at 100 μM were incubated with and without 1 mM AEBSF for 1 h in activity assay buffer containing 10 mM Tris (pH 8.5), 20% glycerol, and 1 mM CHAPS. Excess AEBSF was removed using a NAP-5 column (GE Healthcare). Gel samples were run at 5 μM on an SDS−PAGE gel with and without a reductant to visualize the appearance of the disulfide-bonded band.

Kinetics of Cysteine-Substituted Variants. For kinetic measurements of NS2B-NS3pro activity, 300 nM purified NS2B-NS3pro was monitored over the course of 10 min in an NS2B-NS3pro activity assay buffer containing 10 mM Tris (pH 8.5), 20% glycerol, and 1 mM CHAPS. For substrate titrations, a range of 0−500 μM fluorogenic substrate GRR-AMC [*N*-acetyl-Gly-Arg-Arg-AMC (7-amino-4-methylcoumarin); excitation at 355 nm, emission at 460 nm; Peptides International] was added to start the reaction. Assays were performed in duplicate at 37 °C in 100 μL volumes in 96-well microplate format using a Spectramax M5 spectrophotometric plate reader (Molecular Devices). Initial velocities versus substrate concentration were fit to a rectangular hyperbola using GraphPad Prism (GraphPad Software) to determine kinetic parameters K_M and k_{cat}. Enzyme concentrations were determined by monitoring the absorbance at 280 nm using a nanodrop spectrophotometer (Thermo Scientific). The concentrations were also validated by assessment via Coomassie-stained SDS−PAGE. For enzymes measured with DTT, 10 mM DTT was added to the activity assay buffer with 300 nM enzyme and activity was monitored using 100 μM substrate.

Cross-Linking Cysteine-Substituted Variants. NS2B-NS3pro variants were diluted to 500 μL in 100 mM Tris (pH 7.5) and 100 mM NaCl and treated with the reductant 10 mM tris(2-carboxyethyl)phosphine (TCEP) for 30 min at room temperature. To remove the TCEP, proteins were buffer exchanged into 1× PBS with 5 mM EDTA using a NAP-5 column (GE Healthcare), and bismaleimidoethane (BMOE) was added from 0 to a 100-fold excess with respect to 5 μM protein for 2 h at 4 °C. The reactions were then quenched with 10 mM DTT at room temperature for 15 min before the activity of the NS2B-NS3pro variants was monitored by cleavage of a

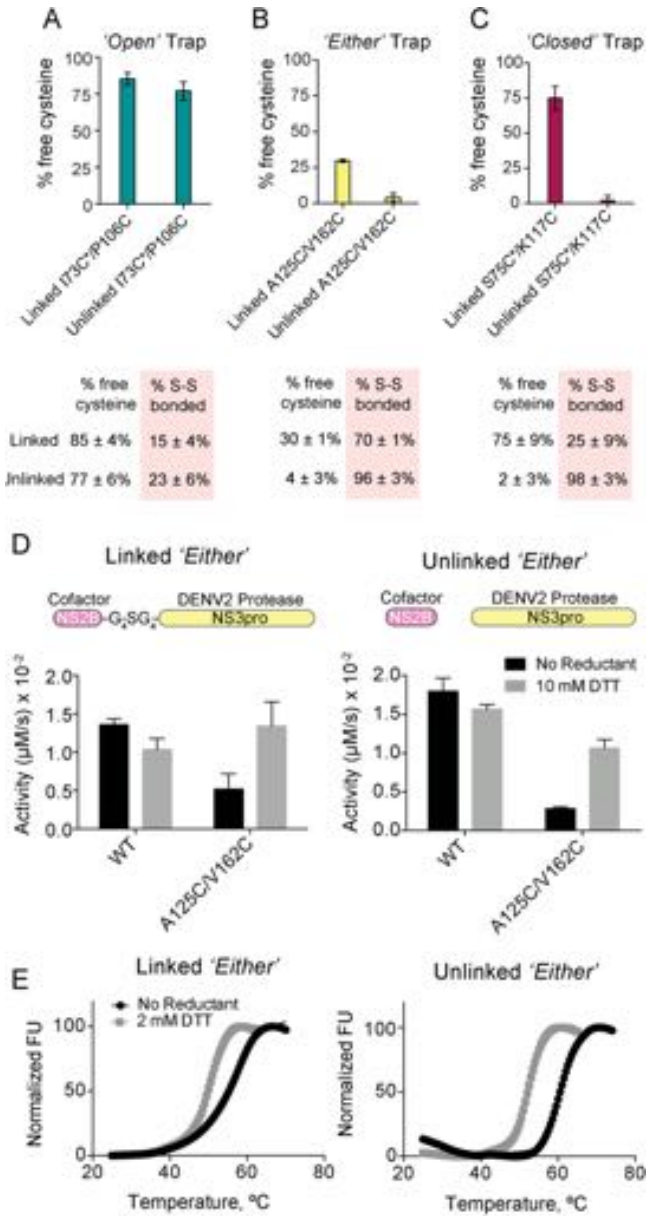

Figure 2. Quantification of free cysteines measures resting linked and unlinked DENV2 conformational states. (A) Both linked and unlinked DENV2 NS2B-NS3pro I73C*/P106C bind DTNB to a similar extent, demonstrating that neither spontaneously samples the "open" conformation to a large extent. (B) Both linked and unlinked DENV2 NS2B-NS3pro A125C/V162C do not bind a large amount of DTNB, suggesting that most of the population is disulfide-bonded. (C) Linked S75C*/K117C binds a significant fraction of DTNB, corresponding to 25% of the enzyme being disulfide-bonded. This is in contrast to the unlinked S75C*/K117C construct, wherein 98% of the enzyme is disulfide-bonded, suggesting that the unlinked version predominantly rests in the "closed" conformation while the linked construct rests in an alternative conformation. (D) Addition of DTT to the linked and unlinked A125C/V162C "either" trap increases activity to near WT levels, suggesting flexibility of the protease core in addition to the NS2B cofactor being required for full activity. The presence of DTT results in a small amount of inhibition. The mechanism of this inhibition is not known. (E) The stability of A125C/V162C was assessed in the presence of DTT or absence of DTT using differential scanning fluorimetry.

DOI: 10.1021/acs.biochem.8b00978
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Table 1. Kinetics of Cysteine Traps and Sensitivity of K117

	linked construct			unlinked construct		
	K_M (μM)	k_{cat} (s^{-1})	k_{cat}/K_M (s^{-1} M^{-1})	K_M (μM)	k_{cat} (s^{-1})	k_{cat}/K_M (s^{-1} M^{-1})
WT	70 ± 9	0.050 ± 0.002	700 ± 90	40 ± 10	0.090 ± 0.008	2000 ± 700
I73C*/P106C	40 ± 8	0.030 ± 0.002	800 ± 200	50 ± 10	0.080 ± 0.005	2000 ± 400
A125C/V162C	30 ± 10	0.020 ± 0.002	800 ± 300	50 ± 7	0.010 ± 0.001	300 ± 40
S75C*/K117C	30 ± 4	0.020 ± 0.001	800 ± 100	40 ± 10	0.070 ± 0.006	2000 ± 800
K117A	40 ± 6	0.006 ± 0.000	200 ± 40	80 ± 10	0.050 ± 0.003	700 ± 100
K117C	50 ± 10	0.020 ± 0.001	440 ± 100			
K117R	50 ± 20	0.040 ± 0.004	800 ± 200			
S75C*	40 ± 4	0.050 ± 0.002	1000 ± 200			

fluorogenic substrate as described above using 300 nM NS2B-NS3pro in 10 mM Tris (pH 8.5), 20% glycerol, and 1 mM CHAPS assay buffer. Cross-linked protein was visualized on nonreducing SDS−PAGE gels in the absence of DTT. Samples with the highest concentration of BMOE (100×) were run with DTT to validate BMOE irreversible cross-linking rather than spontaneous disulfide bond formation that is susceptible to DTT-mediated reduction.

Inhibition by DTNB. The unlinked NS2B-NS3pro A125C cysteine variant (20 μM) and unlinked WT NS2B-NS3pro (20 μM) were each incubated with 200 μM 5,5′-dithiobis(2-nitrobenzoic acid) (DTNB) for 1 h at room temperature, and then activity was monitored. For recovery, the enzymes were diluted to 300 nM and incubated with 5 mM DTT for 30 min at room temperature. Activity was again monitored as described above.

RESULTS AND DISCUSSION

While NS2B-NS3pro from DENV2 has only been observed crystallographically in the "open" (inactive) conformation,[14] its similarity to NS2B-NS3pro from DENV3 and WNV and NMR evidence[18] suggest that it also exists in the "closed" (active/substrate-bound) conformation. To probe the importance of these conformational changes, we devised an experimental approach that allowed NS2B-NS3pro to be trapped in various conformational states. We identified potential pairs of residues that when replaced with cysteine should result in the formation of an intramolecular disulfide in one particular conformation ("open" or "closed"). This method is particularly well-suited for studying DENV2 NS2B-NS3pro as this enzyme is naturally devoid of cysteine residues, which could interfere with the introduced cysteine substitutions. The modeled pairs of cysteine residues were evaluated using MODIP[32] to score the probability of disulfide formation between candidate pairs. Ultimately, we identified and constructed six disulfide pairs (Figure 1C). The I73C*/P106C (the asterisk indicates an NS2B residue) pair is proximal in the "open" conformation but far apart in the "closed" conformation; thus, formation of intramolecular disulfides should trap this variant in the "open" conformation. The A125C/V162C pair is nearly in the same position in the "open" and "closed" states, so intramolecular disulfides are compatible with the "open" or "closed" state and should not affect the mobility of the NS2B cofactor. We therefore call the A125C/V162C variant an "either" trap. The S75C*/K117C pair is close together in the "closed" conformation but far apart in the "open" conformation and should therefore trap this variant in the "closed" conformation upon intramolecular disulfide bond formation. All three traps were made in both the linked construct and the unlinked construct for direct comparison of the conformational flexibility of the enzyme in the presence or absence of the synthetic glycine-rich linker.

To measure the propensity of disulfide bond formation of the various DENV2 NS2B-NS3pro cysteine traps, each construct was purified in the same way in the absence of a reductant during all steps of purification (see WT and Cysteine Variant Expression and Purification). This way, disulfide bonds would readily form only if the enzyme rested in that particular conformation. To quantify these states, the free thiol concentration of the NS2B-NS3pro variants was measured using DTNB, a quantitative, thiol-reactive reagent, and compared to a standard curve using DTT. Significant DTNB absorbance was detected for the "open" conformation in the linked and unlinked constructs (Figure 2A), suggesting that neither the linker nor the unlinked DENV2 NS2B-NS3pro rests naturally in the fully "open" conformation. However, a small portion may sample the "open" state, as the free thiol concentration was determined to be 85 and 77% for linked and unlinked forms, respectively, and not 100% as would be expected if no disulfide bonds formed (Figure 2A). If we presume that disulfide formation is an ideal monitor of the "open" disulfide-bonded populations, this would therefore correspond to 15% for the linked construct and 23% for the unlinked construct (Figure 2A). The "either" trap formed easily for the linked and the unlinked constructs. Specifically, the linked NS2B-NS3pro was 70% disulfide-bonded, whereas the unlinked NS2B-NS3pro was 96% disulfide-bonded (Figure 2B). The most significant difference between the two constructs was the ability to form the disulfide bond in the "closed" (active) conformation. The linked construct did not readily form a "closed" disulfide bond, though it appeared to partially sample the "closed" conformation with 25% of the linked NS2B-NS3pro occupying the "closed" disulfide-bonded state (Figure 2C). It appears that the linked version of NS2B-NS3pro occupies the "open" and "closed" states to a comparable extent, suggesting that the linked construct has a similar propensity to form both the "open" (inactive) conformation or the "closed" (active) conformation. Contrastingly, unlinked NS2B-NS3pro was almost completely captured in the "closed" conformation, with 98% of the enzyme being "closed" disulfide-bonded (Figure 2C). This strongly suggests that the linked and unlinked constructs of DENV2 NS3B-NSpro rest in different conformational states in solution. It appears that the unlinked construct rests in a conformation that is more poised to bind the substrate, which may explain its faster catalytic rate compared to that of the linked construct (Table 1), whereas the equilibrium of the linked construct is shifted to an alternative conformation, likely the "open" conformation. It is important to note that none of the linked double cysteine variants had a major effect on the overall catalytic efficiency of NS2B-NS3pro (Table 1), though a

DOI: 10.1021/acs.biochem.8b00978
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slower k_{cat} was observed for all variants matched by a reduced K_M. The unlinked "open" (I73C*/P106C) and "closed" (S75C*/K117C) double cysteine variants similarly did not affect NS2B-NSpro activity; however, the unlinked "either" trap (A125C/V162C) significantly decreased the catalytic efficiency (Table 1). Because the unlinked "either" trap is almost 100% disulfide-bonded (Figure 2B), our findings suggest that in addition to the mobility of the NS2B cofactor, the flexibility of the entire core of the protein may be important for proteolytic activity. We recognize that if formation of the "either" trap locks DENV2 NS3B-NSpro into an unnatural alternate conformation in the core, that would also lead to inactivation. Our modeling (Figure S1) suggests that an intramolecular disulfide bond could form in the "either" trap (A125C/V162C) without a major distortion to the core of the structure. The degree of structural adjustment required to form a disulfide in the mutant "either" trap can be gleaned from Figure S1. It is our interpretation that the major impact of disulfide bond formation in the either trap would be the decrease in the flexibility of the core, but we cannot rule out the potential that this disulfide bond is negatively impacting activity through structural distortions. Indeed, the addition of DTT to break the disulfide bond between A125C and V162C in the linked and unlinked constructs allowed the enzyme to recover a significant portion of its catalytic activity, suggesting that the individual substitutions alone are not detrimental to catalytic activity and that preventing movement within the core of the protease negatively impacts catalysis by NS2B-NS3pro (Figure 2D). While the A125C/V162C "either" trap does show a 32% reduction in activity due to the presence of the cysteine substitution, the level of reduction radically increases to 84% when intramolecular disulfides are formed. Thus, we see a >50% reduction in activity when the disulfide bonds are formed. The activity can be recovered once the disulfide bonds are broken, in the presence of DTT. This strongly indicates that the disulfide bond is responsible for the majority of the inhibition. A number of studies correlate the formation of disulfide bonds and the loss of intrinsic flexibility, which can be observed as an increase in intrinsic stability. Often, proteins are engineered to be more stable through the introduction of disulfide bonds by rigidifying flexible sites.[33−36] As A125C/V162C is more stable in the oxidized state than in the reduced state (Figure 2E), this strongly supports the idea that the disulfide is rigidifying the protein and limiting the flexibility.

Interpretation of our results hinges on the predominant formation of intramolecular disulfide bonds. To assess the level of intramolecular disulfides formed and simultaneously visualize the effect of disulfide bond formation on protein migration, DENV2 NS2B-NS3pro samples were separated on a nonreducing SDS−PAGE gel in the presence or absence of a reductant (DTT). Linked WT NS2B-NS3pro was unaffected by the presence of a reductant (Figure 3A) because it naturally lacks any cysteine residues. The linked "open" trap (I73C*/P106C) did not form intermolecular disulfide bonds appreciably, as evidenced by the lack of higher-molecular weight bands (Figure 3A). However, a smaller band appeared in the nonreduced lane around 30 kDa, suggesting that disulfide bond formation between NS2B and NS3pro in the linked construct increases the rate of migration of DENV2 NS2B-NS3pro (indicated by NS2B^{S-S}NS3pro) likely due to a decrease in the effective radius of the protein (Figure 3A). The linked "either" trap (A125C/V162C) also did not appear to form intermolecular disulfide bonds (Figure 3A). The absence of the

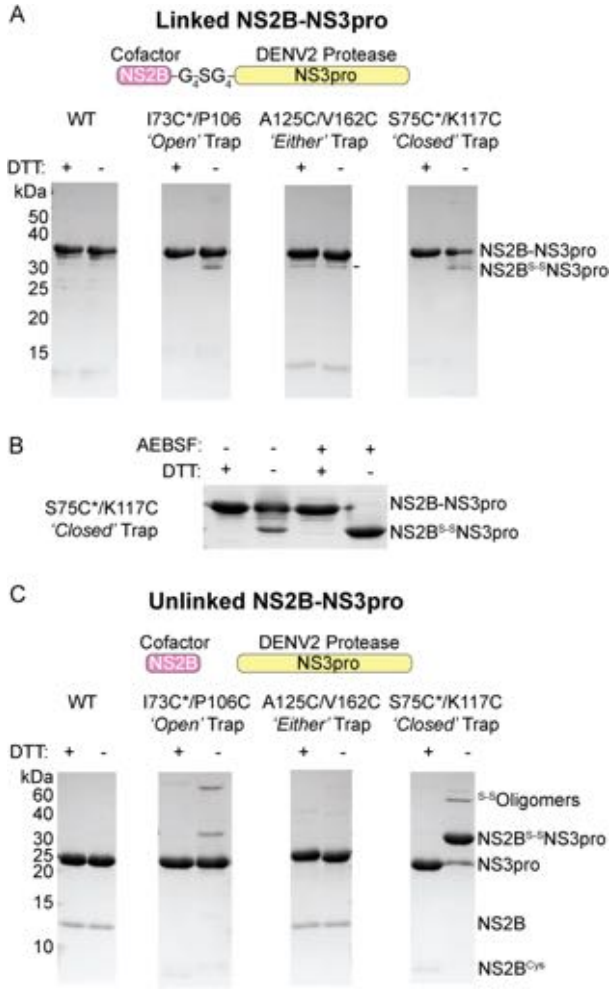

Figure 3. Visualization of disulfide-bonded variants on a nonreducing SDS−PAGE gel. (A) Migration of linked DENV2 NS2B-NS3pro variants. Linked DENV2 NS2B-NS3pro WT migrates similarly in the presence or absence of DTT. Linked I73C*/P106C forms the NS2B^{S-S}NS3pro band in the absence of DTT that migrates as a 30 kDa band. Linked A125C/V162C, in which both mutations are within the protease domain, does not migrate differently without DTT. While some light bands are observed beneath the NS2B-NS3pro bands in the "either" variant, their presence in the reduced lane suggests that these bands do not arise via disulfide bond formation and could be either cleaved versions of NS2B-NS3pro or contaminants [represented by a dash (−)]. Linked S75C*/K117C, like I73C*/P106C, forms the NS2B^{S-S}NS3pro band at 30 kDa in the absence of DTT. The full height of the gel is shown to enable assessment of purity. (B) Linked S75C*/K117C is induced to form the NS2B^{S-S}NS3pro "closed" conformation in the presence of active site inhibitor AEBSF, suggesting that these mutations form a disulfide bond when the enzyme is resting in the "active" conformation. (C) Migration of unlinked DENV2 NS2B-NS3pro variants. Unlinked WT DENV2 NS2B-NS3pro migration is unchanged in the presence of DTT. Unlinked I73C*/P106C forms a small amount of NS2B^{S-S}NS3pro, which shows retarded migration as NS2B and NS3pro electrophorese as a single disulfide-bonded complex. Unlinked A125C/V162C does not migrate differently without DTT because both cysteine insertions are in the protease domain. Unlinked S75C*/K117C predominantly runs as NS2B^{S-S}NS3pro, supporting the consensus that the unlinked construct rests in the "closed" conformation.

smaller disulfide-bonded band is likely due to the fact that both cysteine substitutions are within the protease domain, are

DOI: 10.1021/acs.biochem.8b00978
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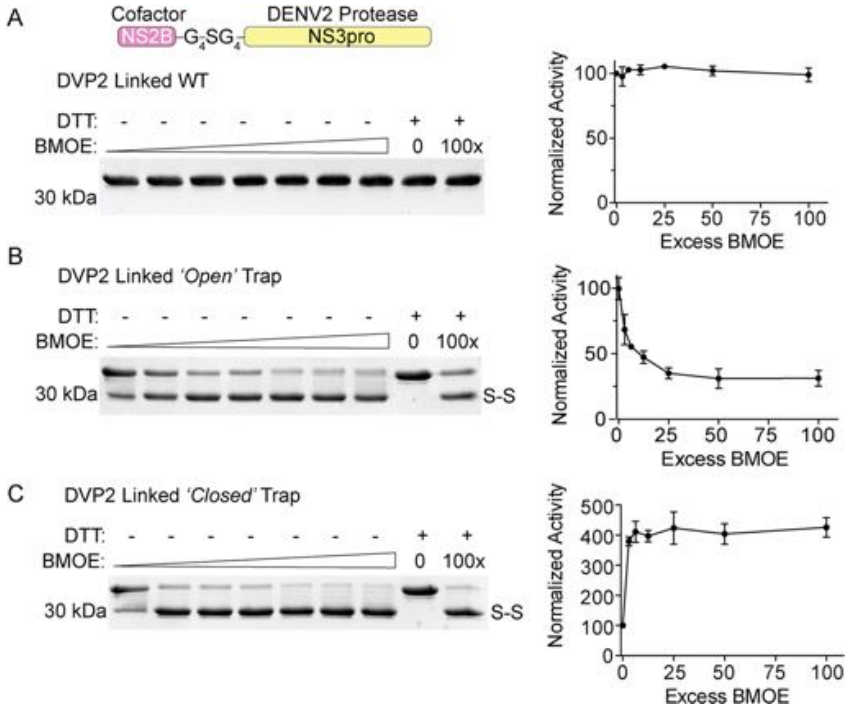

Figure 4. Linked DENV2 NS2B-NS3pro cysteine variants can be cross-linked by BMOE to form the "open" (inactive) or "closed" (active) conformation. Cross-links were introduced using the short-arm cross-linker BMOE (bismaleimidoethane). (A) Linked WT DENV2 NS2B-NS3pro does not migrate differently with BMOE on an SDS−PAGE gel in the absence of a reductant. Its activity is also unaffected by a ≤100-fold excess of BMOE. (B) The linked DENV2 NS2B-NS3pro "open" trap (I73C*/P106C) is readily cross-linked by excess BMOE, corresponding to a decrease in catalytic activity. At a 100-fold excess of BMOE, the "open" trap is 61% cross-linked, which corresponded to a 69% decrease in activity. (C) The linked DENV2 NS2B-NS3pro "closed" trap (S75C*/K117C) is easily cross-linked by excess BMOE. At a 100-fold excess of BMOE, the "closed" trap is 83% cross-linked, which corresponded to a 425% increase in activity.

closer to each other in sequence, and are not predicted to impact the overall global structure of the enzyme. The linked "closed" trap (S75C*/K117C) did not readily form intermolecular disulfide bonds, but a portion show a migration shift to the NS2B^{S-S}NS3pro band in the absence of DTT, as well (Figure 3A). This population of S75C*/K117C could be nearly completely shifted to the disulfide-bonded band after adding the serine protease inhibitor, AEBSF (Figure 3B), suggesting that active site occupancy induces the "closed" conformation. In contrast, in the I73C*/P106C open trap, a significantly smaller fraction of total protein was observed in NS2B^{S-S}NS3pro in the presence of AEBSF (Figure S2), underscoring the fact that an active site inhibitor does not promote the open conformation but supports the closed conformation. The linked "open" and linked "closed" traps each have one cysteine substitution in the NS2B cofactor region (I73C* and S75C*, respectively) and one cysteine substitution in the NS3pro domain (P106C and K117C, respectively), so it is expected that each should shift similarly on a nonreducing SDS−PAGE gel when disulfide-bonded. These results for the linked "open" and linked "closed" traps are also consistent with the quantification (Figure 2A), wherein the linked constructs show similar likelihoods of forming the "open" or "closed" conformation.

Different results are observed for the unlinked constructs. Unlinked NS2B-NS3pro WT migrates as two independent bands because the NS2B cofactor and NS3pro are co-expressed and are not covalently linked (Figure 3C). Again, WT was unaffected by the presence or absence of DTT, because no cysteine residues are present (Figure 3C). In the presence of DTT, the unlinked "open" trap (I73C*/P106C) NS3pro migrates the same as WT, whereas the NS2B^{Cys} cofactor runs much lower and is observed as a very faint band. Regardless, in the absence of DTT, a small portion of the NS2B cofactor and the NS3pro region form disulfide bonds and migrate as a single band that coincides with the addition of their individual molecular weights, also denoted NS2B^{S-S}NS3pro (Figure 3C). In this case, a small amount of intermolecular disulfide bonds also forms, indicated by the formation of oligomers during electrophoresis. The unlinked "either" trap (A125C/V162C) does not form intermolecular disulfide bonds (Figure 3C), but the intramolecular disulfide bond in this particular variant does not change the migration of the NS2B or NS3pro bands in the absence of DTT. This is because both cysteine substitutions are in the protease domain, so formation of a disulfide bond would not cause the NS2B cofactor and NS3pro region to be covalently bonded. The unlinked "closed" trap (S75C*/K117C) behaves like the "open" trap (I73C*/P106C) in the presence of DTT (Figure 3B), as both bear a cysteine substitution in a similar location of the NS2B cofactor. However, in the absence of DTT, a predominant portion shifts to the NS2B^{S-S}NS3pro band, showing that the "closed" disulfide bond is easily formed (Figure 3C), which is also consistent with the DTNB quantification indicating protected cysteines (Figure 2C). In this case, there is also a small amount of intermolecular disulfide bonds that form, as well, indicated by the higher band on the gel. However, it is obvious that the majorly preferred state is in a monomeric disulfide-bonded conformation. These gels clearly show that the equilibria between the linked construct and the unlinked construct are different: the linked construct rests in an intermediate conformation sampling the "open" and "closed" states, whereas the

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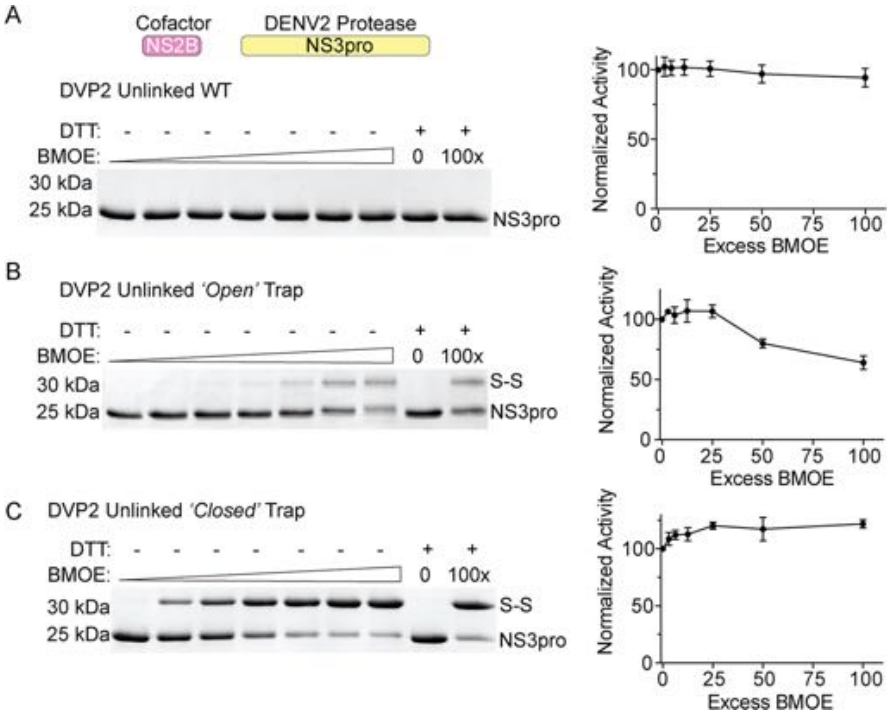

Figure 5. Unlinked DENV2 NS2B-NS3pro can form "open" and "closed" conformations in the presence of BMOE. Cross-links were introduced using the short-arm cross-linker BMOE (bismaleimidoethane). (A) Unlinked DENV2 NS2B-NS3pro WT migration is unaffected by the presence of BMOE on an SDS−PAGE gel in the absence of the reductant. Its activity is also unaffected by a ≤100-fold excess of BMOE. (B) The unlinked DENV2 NS2B-NS3pro "open" trap (I73C*/P106C) can be cross-linked with a large excess of BMOE, corresponding to a decrease in activity. With a 100-fold excess of BMOE, the "open" trap is 35% cross-linked, which led to a 36% decrease in activity. (C) The unlinked DENV2 NS2B-NS3pro "closed" trap (S75C*/K117C) is easily cross-linked by excess BMOE. At a 100-fold excess of BMOE, the "closed" trap is 72% cross-linked, which increased the catalytic activity by 22%.

unlinked construct predominantly rests in the "closed" conformation.

To lock the enzyme into distinct conformations, the irreversible short-arm sulfhydryl to sulfhydryl cross-linker, bismaleimidoethane (BMOE), was used to conjugate the two introduced cysteines. Irreversibly cross-linking the introduced cysteines allows one to probe multiple aspects, including the ease of cross-link formation and the effect on activity. The linked NS2B-NS3pro WT "open" and "closed" traps were each titrated with excess BMOE (panels A−C of Figure 4, respectively). The BMOE cross-linking reaction occurs in the presence of NaCl concentrations that are inhibitory to DENV2 NS2B-NS3pro.[17,24] Nevertheless, because the same conditions were used for all BMOE assessments, we anticipate that meaningful comparisons can be drawn from these studies. Linked WT NS2B-NS3pro, lacking any cysteine residues, was not inhibited by increasing concentrations of BMOE (Figure 4A); likewise, its migration on a nonreducing SDS−PAGE gel was also unaffected (Figure 4A). The linked "open" trap was successfully cross-linked, visualized by the appearance of the faster-migrating band on a nonreducing SDS−PAGE gel (absence of DTT) (Figure 4B). Upon addition of the reductant, the sample lacking BMOE migrates at the expected size without any disulfide-bonded population. To verify that the lower-molecular weight band was due to the BMOE cross-linker, the highest concentration of BMOE (100-fold excess) in the presence of DTT was also analyzed. Because the lower-molecular weight band is still present with DTT, it suggests that the disulfide bond formed is due to the treatment with BMOE, which is an irreversible cross-linker. Additionally, the linked NS2B-NS3pro "open" trap was inhibited by increasing amounts of BMOE, further demonstrating that the "open" conformation is indeed an inactive conformation (Figure 4B). At a 100-fold excess of BMOE, the "open" trap is 61% cross-linked, which corresponded to a 69% decrease in activity. The linked NS2B-NS3pro "closed" trap was also successfully cross-linked, again visualized by the appearance of a smaller band on an SDS−PAGE nonreducing gel (Figure 4C). However, in this case, the activity of the cross-linked "closed" trap was significantly (>400%) higher in the presence of BMOE (Figure 4C). This result supports the case that keeping the NS2B cofactor in the proximity of the active site increases the enzyme's ability to turn over the substrate. In summary, cross-linking studies using the linked construct verify the "open" state as being inactive and further suggest that preserving the "closed" state is activating. In addition, both states were successfully trapped to a similar extent (69% for the "open" state and 83% for the "closed" state), suggesting that the linked construct rests in an intermediate state between these two strikingly different conformations.

The unlinked construct was also titrated with BMOE to monitor the propensity of this unlinked construct to adopt the "open" and "closed" conformations. As expected because of its lack of native cysteine residues, neither the activity of unlinked WT NS2B-NS3pro nor its migration on a nonreducing SDS−PAGE gel was impacted by addition of BMOE (Figure 5A). Interestingly, when the same assay was performed on the unlinked "open" trap, cross-linking between the two domains could be observed ($NS2B^{S-S}NS3pro$ disulfide-bonded band) at higher concentrations of BMOE. This cross-linking correlated to a loss of catalytic activity (Figure 5B). This result suggests

DOI: 10.1021/acs.biochem.8b00978
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that, although the enzyme rests in the "closed" state, it remains flexible and is able to sample the "open" conformation. The unlinked "closed" trap readily formed in the presence of all excess amounts of BMOE; however, the activity increased only slightly (Figure 5C). This further suggests that the unlinked construct rests in a state similar to that of the BMOE cross-linked "closed" conformation. In summary, cross-linking studies reveal that while the unlinked construct is more easily trapped in the "closed" conformation, it can also be trapped in the "open" conformation, suggesting that the "open" conformation may be relevant for drug discovery. However, the "closed" conformation is a better representation of the native resting state of the unlinked construct. This is in contrast to the linked construct that shows a significantly altered conformational equilibrium.

We next sought additional means of evaluating whether the conformational equilibrium between the linked and unlinked constructs was significantly different. While choosing residues for the cysteine trap designs, we observed a single substitution, K117A, which in the linked construct showed a significantly lowered k_{cat}, 7-fold lower than that of the WT. In comparison, the unlinked K117A variant had a k_{cat} value only 1.5 times lower than that of the WT (Table 1). As residue 117 is surface-exposed and does not make any visible contacts in either the DENV2 "open" crystal structure or the DENV3 "closed" crystal structure, this result was surprising. The differences in response to the introduced K117A substitution further support the idea that the linked NS2B-NS3pro rests in a state different from that of unlinked NS2B-NS3pro. From these results, it is important to note that substitutions that affect one construct may not necessarily affect the other, and as such, it is essential to remember that data between the two constructs are independent and should not be interchanged. These findings and the fact that the unlinked construct mirrors more closely the virally expressed and processed enzyme suggest that the unlinked construct should be used in future studies of DENV and related NS2B-NS3 proteases.

To further probe the sensitivity of K117 in the linked construct, we produced the single substitutions K117C and K117R in linked DENV2 NS2B-NS3pro. K117C was more active than K117A though still less active than WT, whereas K117R showed activity similar to that of the WT (Table 1). Clearly, a positive charge is important for activity in the linked construct. Single variant S75C* appears to enhance activity, which has been observed before in the DENV2 unlinked construct.[18] One hypothesis explaining K117 sensitivity is that this residue is important for movement of the loops above the A125 allosteric site (Figure 6A,B). The A125 site has been found to be functional in linked DENV2,[37] a split luciferase version of DENV2,[38] and unlinked ZIKV NS2B-NS3pro.[39] To investigate whether the A125 site is functional in unlinked DENV2 NS2B-NS3pro, the A125C variant was produced and reacted with the cysteine-reactive small molecule DTNB. Consistently, the WT was not inhibited by DTNB whereas the A125C variant was completely inhibited (Figure 6C). This inhibition could be relieved by the addition of the reductant DTT. This result is very telling because A125 is inaccessible in the "closed" conformation (Figure 6). The fact that unlinked DENV2 NS2B-NS3pro can be inhibited by DTNB at A125C suggests that the "closed" conformation is not the only relevant conformation for rational inhibitor design and that both "closed" and "open" conformations are represented in the native DENV2 NS2B-NS3pro equilibrium.

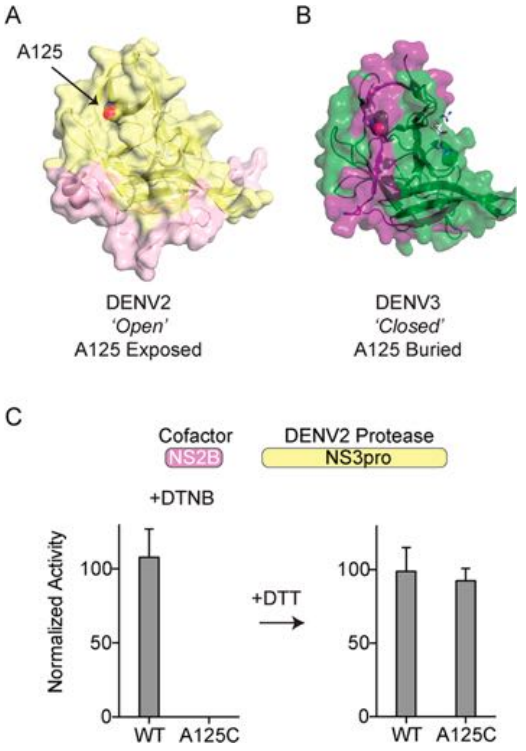

Figure 6. Unlinked DENV2 NS2B-NS3pro is allosterically inhibited at residue A125, which is not accessible in the "closed" conformation. (A) Transparent surface representation of DENV2 NS2B-NS3pro (NS2B colored pink, NS3pro colored yellow) with A125 highlighted. A125 is exposed in this "open" conformation. (B) Transparent surface representation of DENV3 NS2B-NS3pro (NS2B colored purple, NS3pro colored green) with A125 completely buried by the NS2B cofactor in the "closed" conformation. (C) Unlinked WT DENV2 NS2B-NS3pro activity is unaffected by DTNB, whereas the A125C variant is completely inhibited by DTNB. This inhibition can be reversed upon the addition of the reductant DTT. These data suggest that the enzyme is not "locked" in the "closed" conformation in solution but is dynamic enough to allow DTNB to react with A125C.

Though DENV2 NS2B-NS3pro is an excellent target for antiviral drug development, the relevance of certain conformations observed crystallographically has been called into question. In this work, we have developed methods that allow us to quantify the various states of dengue virus protease in solution by capturing multiple conformations using cysteine traps. The fact that forming the "open" trap by cross-linking of I73C*/P106C inactivates NS2B-NS3pro is consistent with prior data and the prevailing paradigm, indicating that the "open" conformation is an inactive conformation and NS2B-NS3pro must adopt the "closed" conformation to bind and turn over the substrate. The presence of a synthetic linkage in the linked construct appears to distort the equilibrium by promoting the "open" conformation, although the unlinked construct retains the ability to sample the "open" conformation to some extent (Figure 7). For this reason, the "open" conformation should not necessarily be disregarded for drug development. The finding that formation of the A125C/V162C disulfide is capable of inactivating NS2B-NS3pro is more surprising. The modeled geometry of this disulfide is very similar to that observed in the "open" and "closed" forms of NS2B-NS3pro (Figure S1), requiring a movement of just 2 Å to achieve the disulfide-bonded conformation. This suggests that in addition to the "closed" conformation, rigidification of the protein core

DOI: 10.1021/acs.biochem.8b00978
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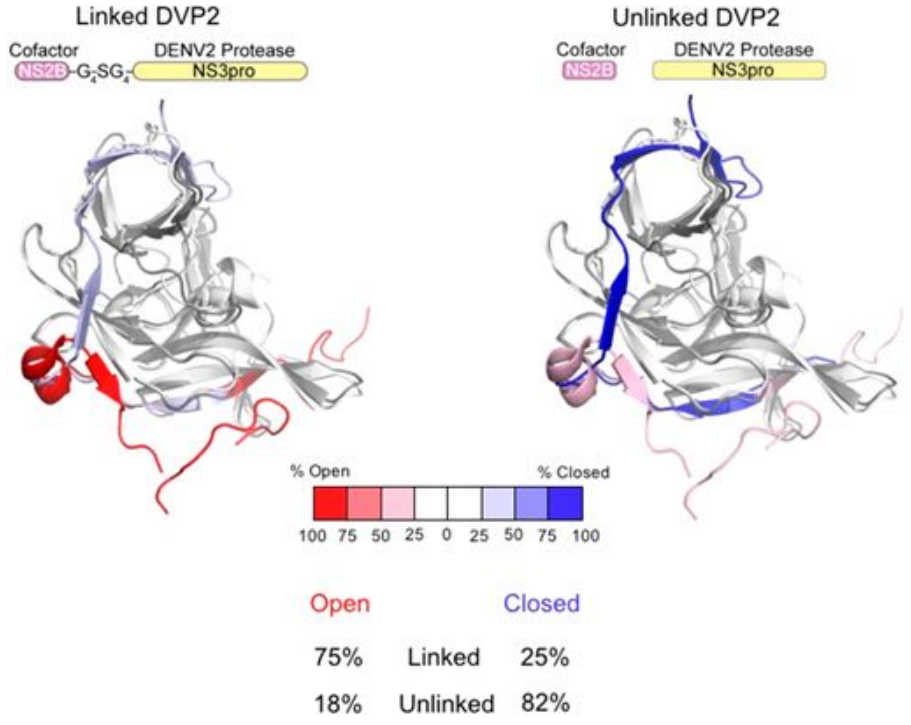

Figure 7. Linked and unlinked DENV2 NS2B-NS3pro show different conformational ensembles. The superimposed structures of the "open" and "closed" conformations of DENV2 NS2B-NS3 are shown. The NS2B regions are colored according to the scale shown, to represent the percentage of each state that is present in the conformational ensemble of the linked and unlinked constructs, respectively. The NS3 regions of the "open" (light gray) and "closed" (gray) conformations are shown. The percent in each conformation was calculated using the increase in activity upon addition of BMOE to the "closed" trap, as shown in Figures 4 and 5. As the "closed" trap is the active conformation, a 4-fold increase in activity after the addition of BMOE in the linked construct suggests that only 25% is resting in the "closed" conformation. However, a modest 1.22-fold increase in activity after addition of BMOE in the unlinked construct suggests that 82% of the enzyme is resting in the closed conformation.

in the A125/V162 region by the presence of a disulfide is likewise inactivating. Clearly, NS2B-NS3pro is a very flexible enzyme, given the large conformational changes that can and do occur in the NS2B region. Our findings indicate that flexibility of the entire core of the protein may be required for the activity of NS2B-NS3pro. This observation is relevant in selecting the types of inhibitors that may be useful for targeting NS2B-NS3pro in dengue-related therapies.

While this work sheds light on the DENV2 NS2B-NS3pro conformational equilibrium, it is critical to recall that each flavivirus protease may behave differently. For instance, ZIKV NS2B-NS3pro is significantly more active than DENV2 NS2B-NS3pro.[16,39] Consistently, the linked ZIKV NS2B-NS3pro construct is less active than the unlinked version[40,41] but has been crystallized in numerous conformations, whereas the unlinked construct has been crystallized in only the "closed" conformation.[27,42,43] In solution, a linked ZIKV NS2B-NS3pro construct assumes an open conformation in the absence of an inhibitor.[44] Clearly, more structural studies of the entire flavivirus protease family as a whole are needed, particularly more solution-based studies such as the one presented here. Regardless, it is clear that the flexibility of the flavivirus proteases is important to their function, but how we are able to harness this feature for druggability remains a challenge.

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acs.biochem.8b00978.

Figures S1 and S2 (PDF)

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Funding

This work was supported by National Science Foundation Grant CBET 1511367 to J.A.H.

Notes

The authors declare no competing financial interest.

■ ACKNOWLEDGMENTS

The authors thank Yunlong Zhao for mass spectrometry measurements on an earlier version of this project, which were not included in this work.

■ ABBREVIATIONS

DENV, dengue virus; NS2B-NS3pro, NS2B-NS3 protease; WNV, West Nile virus; ZIKV, Zika virus; aa, amino acids; DTNB, 5,5-dithiobis(2-nitrobenzoic acid); DTT, dithiothreitol; BMOE, bismaleimidoethane; PDB, Protein Data Bank.

DOI: 10.1021/acs.biochem.8b00978
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DOI: 10.1021/acs.biochem.8b00978
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