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Hepatitis C Virus NS3 Protease Is Activated by Low Concentrations of Protease Inhibitors

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ABSTRACT: The nonstructural protein 3 (NS3) of hepatitis C virus (HCV) is a bifunctional enzyme with a protease and a helicase functionality located in each of the two domains of the single peptide chain. There is little experimental evidence for a functional role of this unexpected arrangement since artificial single domain forms of both enzymes are catalytically competent. We have observed that low concentrations of certain protease inhibitors activate the protease of full-length NS3 from HCV genotype 1a with up to 100%, depending on the preincubation time and the inhibitor used. The activation was reduced, but not eliminated, by increased ionic strength, lowered glycerol concentration, or lowered pH. In all cases, it was at the expense of a significant loss of activity. Activation was not seen with the artificial protease domain of genotype 1b NS3 fused with a fragment of the NS4A cofactor. This truncated and covalently modified enzyme form was much less active and exhibited fundamentally different catalytic properties to the full-length NS3 protease without the fused cofactor. The most plausible explanation for the activation was found to involve a slow transition between two enzyme conformations, which differed in their catalytic ability and affinity for inhibitors. Equations derived based on this assumption resulted in better fits to the experimental data than the equation for simple competitive inhibition. The mechanism may involve an inhibitor-induced stabilization of the helicase domain in a conformation that enhances the protease activity, or an improved alignment of the catalytic triad in the protease. The proposed mnemonic mechanism and derived equations are viable for both these explanations and can serve as a basic framework for future studies of enzymes activated by inhibitors or other ligands.

Hepatitis C virus (HCV)[1] nonstructural protein 3 (NS3) is a protease/helicase/NTPase vital for viral proliferation (for a review, see ref *1*). NS3 protease has been validated as a drug target and several compounds targeting the enzyme are currently in clinical trials (for a review, see ref *2*). The different functions and the complex structure of NS3 also make it an interesting enzyme to study from a biochemical point of view.

Overexpression of full-length NS3 in *Escherichia coli* results in low yields of pure enzyme. It is unstable and partially unfolded in the absence of the viral NS4A protein, which acts both as a membrane anchor and as a cofactor crucial for NS3 protease activity (*3*, *4*). To overcome these problems, many research groups have adopted strategies where only one of the two domains of NS3 is expressed. In addition, NS4A, or a fragment thereof, is often coexpressed with NS3 (*5*−*8*).

Although the two domains of NS3 are functionally independent and truncated forms are catalytically competent, numerous studies have shown how the two domains of NS3 can influence one another (*8*−*17*). The crystal structure of full-length NS3 shows the helicase domain positioned in close proximity to the active site of the protease, essentially serving as a lid (*18*). Interestingly, a recent study of the membrane association of full-length NS3 suggests the helicase domain to be positioned far away from the protease active site, leaving it uncovered (*19*). Although these different conformations may well represent functionally relevant structures in different stages of the viral life cycle, experimental evidence for such mobility of the two domains is lacking.

In order to understand the properties of native NS3 and to find potent inhibitors against the physiologically relevant form of the protease, all our studies have been performed with full-length HCV NS3 from genotype 1a without coexpressed NS4A, despite the low yield and poor stability of this enzyme form (*11*, *16*, *20*−*27*). Instead, a truncated form of NS4A has been added separately. It corresponds to the part of NS4A that is required to activate NS3 in vitro (*28*) and to which lysines have been introduced to increase its solubility (*10*).

During our work with full-length HCV NS3, we have discovered that some protease inhibitors, at low concentration, activated the enzyme. This has not, to our knowledge, been reported or investigated before. However, in light of recent studies suggesting flexibility in NS3 and a role of the helicase domain for protease functionality, the activation was recognized to be of potential functional relevance. It was therefore the focus of the present study.

MATERIALS AND METHODS

Reagents. HEPES (4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid), MES (2-(*N*-morpholino)ethanesulfonic acid), dimethyl sulfoxide (DMSO) (Sigma-Aldrich Sweden AB, Stockholm, Sweden), glycerol (Merck KgaA, Darmstadt, Germany),

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[1]Abbreviations: CHAPS, 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate; DMSO, dimethylsulfoxide; flNS3, full-length NS3 protein; HCV, hepatitis C virus, NS3, nonstructural protein 3.

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3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate (CHAPS, Anatrace, Maumee, OH, USA), *n*-octyl-β-D-glycopyranoside (Anatrace, Maumee, OH, USA), and DTT (dithiothretiol, Sigma-Aldrich Sweden AB, Stockholm, Sweden) were taken from new containers.

Enzymes and NS4A Cofactor. Expression and purification of full-length HCV NS3 from genotype 1a, denoted flNS3 throughout the paper, were performed as described previously (*16*, *26*). This construct contains an N-terminal tag of 38 amino acids, including a hexa-histidine sequence. All experiments presented in this paper were performed using the same enzyme batch, although flNS3 from different batches has been tested to verify that the results were not batch dependent. Recombinant truncated HCV NS3/4A protease from strain 1b, with a short NS4A peptide fused to the NS3 N-terminus, was purchased from AnaSpec, San Jose, CA, USA. This construct contains the central part of the NS4A cofactor peptide fused to the N-terminus of the NS3 protease domain (amino acids 1−181) through a short GSGS-linker. This protein is denoted NS3/4A throughout the paper.

The NS4A cofactor peptide, KKGSVVIVGRIVLSGK, referred to as NS4A throughout the paper, was obtained from Gunnar Lindeberg at the Department of Medicinal Chemistry, Uppsala University. It was freshly diluted in DMSO before use.

Inhibitors and Substrate. Inhibitors (Figure 1), obtained from the Department of Medicinal Chemistry, Uppsala University (compounds 1−10), Boehringer-Ingelheim, (BILN 2061) and the VIRGIL DrugPharm Team (VX 950) were also freshly diluted in DMSO. The FRET substrate RET S1, based on the NS4A-4B cleavage site and which gives rise to a fluorescence signal once cleaved (Ac-Asp-Glu-Asp(EDANS)-Glu-Glu-Abu-ψ-[COO]Ala-Ser-Lys(DABCYL)-NH_2 (AnaSpec, San Jose, CA, USA)), was freshly diluted in assay buffer (50 mM HEPES, pH 7.4, 40% (w/v) glycerol, 0.1% (w/v) *n*-octyl-β-D-glycopyranoside and 10 mM DTT, 3.33% (v/v) DMSO).

NS3 Protease Activity Measurements. The NS3 protease activity measurements involved the preincubation of 1 nM flNS3 and 25 μM NS4A peptide in assay buffer, in a total volume of 290 μL, for 15 min at 30 °C. The reaction was started by addition of 10 μL of substrate and thorough mixing. The final volume in each well after mixing was 235 μL. The reaction was monitored using a fluorescence plate reader (Fluoroskan Ascent Labsystems, Stockholm, Sweden) and the change in fluorescence per second was measured. The initial rates were determined using the first, linear phase of the progress curves. This procedure is hence referred to as the standard assay procedure. All measurements were performed at least in triplicates. Most initial rates were simply normalized and simply used for comparative analysis. When necessary, corrections for the inner-filter effect was performed (*29*), and the units were converted to μM/s. Enzyme stability was analyzed by preincubating 1 nM flNS3 and 25 μM NS4A in assay buffer between 10 and 95 min before starting the reaction by addition of 0.5 μM substrate.

Inhibition/Activation Measurements. Analysis of the inhibition/activation of flNS3 by the inhibitors was performed with the standard assay, allowing the inhibitor to preincubate with NS3 and NS4A for 15 min prior to addition of substrate. A final substrate concentration of 0.5 μM and inhibitor concentrations between 0 to 60 μM was used for all inhibition/activation measurements. K_i-values were determined by fitting eq 1 or 4 to the initial rates (see below).

The time dependency for the activation was assayed by preincubating 0.2 μM of inhibitor **2** with 1 or 2 nM flNS3 and 25 μM NS4A in assay buffer for varying times up to 30 min before starting the measurement by adding 0.5 μM substrate. The time dependency for the slow inhibition of VX 950 was assayed by preincubating 2.5 or 5 nM inhibitor with 1 nM flNS3 and 25 μM NS4A in assay buffer for up to 45 min before starting the measurements by adding 0.5 μM substrate.

The Effect of Activation on the Catalytic Properties of NS3 Protease. Substrate saturation experiments in the presence or absence of 0.2 μM inhibitor **2** were performed using the standard assay procedure with substrate concentrations ranging from 0.125 to 10 μM, and fitting the Michaelis−Menten equation to the initial rates. The effect of varying NS4A concentrations was investigated in the absence or presence of 0.2 μM inhibitor **2** by preincubating 1 nM flNS3 and up to 40 μM NS4A in assay buffer for 15 min before starting the reaction by adding 0.5 μM substrate.

Forces Behind the Activation. To see if the inhibitor could activate flNS3 in the absence of NS4A, 1 nM flNS3 was preincubated with 0.2 μM inhibitor **2** in assay buffer for 15 min prior to adding 25 μM NS4A, mixing, adding 0.5 μM substrate, mixing and measuring. Similarly, to see if the inhibitor affected only NS4A, 25 μM cofactor was preincubated for 15 min with 0.2 μM inhibitor **2** in assay buffer prior to adding 1 nM flNS3, mixing, adding 0.5 μM substrate, mixing and measuring.

Effects of activation of flNS3 due to changes in buffer conditions was investigated by preincubating 1 nM flNS3, 25 μM NS4A and 0−3 μM inhibitor **2** in assay buffer without glycerol, or high ionic strength (up to 1 M NaCl), or pH 6.0 (using 50 mM MES instead of HEPES) or with 0.1% CHAPS instead of 0.1% *n*-octyl-β-D-glycopyranoside for 15 min before adding 0.5 μM substrate and measuring NS3 protease activity.

Determination of Kinetic Model. The equations describing the activation effect (eq 1−3) were derived on the basis of an enzyme mnemonic process (*30*, *31*) (see Supporting Information). The suitability of the different models and the kinetic parameters were determined using nonlinear regression analysis (STATISTICA, StatSoft, Tulsa, OK, USA) or simulated annealing procedures (GOSA, Bio-Log, Ramonville, France).

Truncated NS3/NS4A. Kinetic parameters for truncated NS3/NS4A were determined using up to 8 μM substrate and the standard assay procedure with an assay buffer supplemented with 600 mM NaCl. Activation/inhibition of NS3/NS4A by inhibitor **2** was performed by preincubating up to 30 μM inhibitor with 1 or 2 nM NS3/NS4A, with or without 25 μM NS4A, in assay buffer containing no glycerol, or up to 1 M NaCl, or pH 6.0 or CHAPS instead of *n*-octyl-β-D-glycopyranoside for 15 min before adding 0.5 or 2 μM substrate.

RESULTS

Optimization of Procedures for Investigating Enzyme Activation. A preliminary analysis of the characteristics of the inhibitors in our library revealed that some inhibitors activated the enzyme when added at low concentrations and were only inhibitory at higher concentrations. The classification was defined on the basis of the initial rates, measured after 10 min preincubation of enzyme, cofactor, and inhibitor, and with the reaction initiated by addition of substrate. Inhibitors were defined as being "activating" if the protease activity was higher in the presence of low inhibitor concentrations than in the absence of inhibitor. A set of inhibitors representing these two

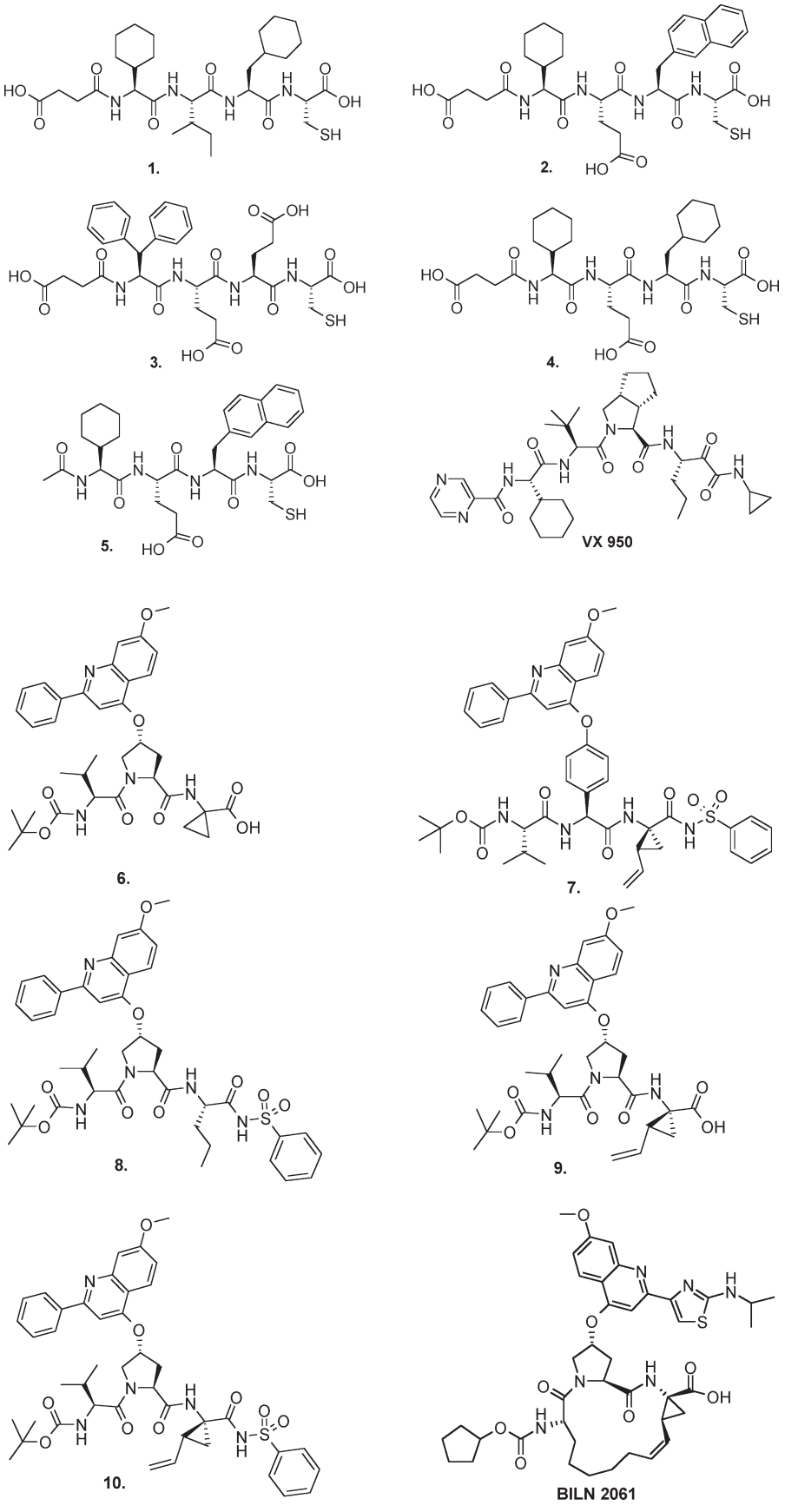


FIGURE 1: Structures of the studied inhibitors, with compounds containing small and large P2 side-chains.

classes was chosen for more extensive studies (Figure 1). They were selected to represent a broad range of structures and potencies, from sub-nanomolar to micromolar K_i-values (*16*, *21*, *26*, *27*). Since it was not practical (or necessary) to test every condition for every inhibitor, inhibitor **2** was chosen for more in-depth studies of the activation phenomenon. It was selected since it clearly

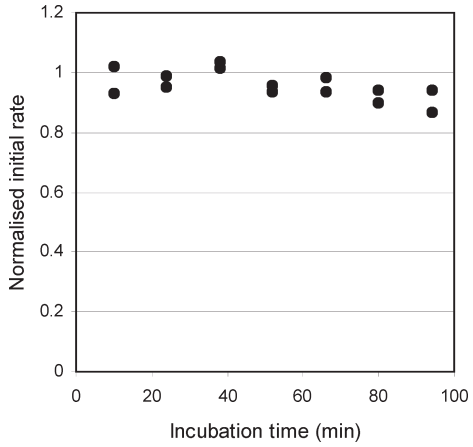


FIGURE 2: Stability of flNS3 under standard assay conditions. 1 nM flNS3 and 25 μM NS4A were preincubated at 30 °C between 10 and 90 min in 50 mM HEPES pH 7.4, 40% (w/v) glycerol, 0.1% (w/v) *n*-octyl-β-D-glycopyranoside and 10 mM DTT and 3.3% (v/v) DMSO before the reaction was started by addition of substrate. Initial rates were normalized to activities for preincubation at t = 10 min.

activated NS3 (up to 40% using 15 min preincubation) and that its highest activating concentration was low (0.2 μM), allowing the same stock solution of inhibitor to be used for all studies.

Before the nature of the HCV flNS3 activation by low concentrations of certain inhibitors was explored, trivial practical reasons for the activation were excluded. Effects caused by faulty reagents or spurious batches of NS3 were eliminated by the use of fresh solutions and verification of the results with enzyme from a different batch. Pipettes and instrumentation were also thoroughly checked for inconsistencies and errors. The procedures for mixing and measuring NS3 serine protease activity were optimized to minimize errors and to allow the addition of substances in any order. Progress curves were recorded for periods between 1 and 10 min depending on the assay conditions used, never allowing more than 10% substrate conversion. The enzyme was not self-degrading to any significant degree within the time frame used for the protease activity measurements, with less than 10% loss in activity after 90 min at 30 °C (Figure 2).

Activation Is Dependent on Preincubation. Activation was only seen when the inhibitors were preincubated with flNS3 and NS4A prior to addition of substrate. The magnitude of the activation was found to be 20 to 100% depending on the inhibitor and preincubation time. Preincubation of 0.2 μM inhibitor **2** with 1 nM enzyme resulted in maximum activation after about 17 min of preincubation, with 60% higher protease activity than without preincubation (Figure 3A). Increasing the enzyme concentration to 2 nM increased both the time required to reach maximal activation (to around 25 min) and the activation reached (to 100%) (Figure 3A). On the basis of this, the preincubation time for assaying activation with the selected compounds was set to 15 min and the enzyme concentration was set to 1 nM. By using a longer preincubation time, compounds that had previously shown no sign of activation using 10 min preincubation, which was our previous standard, now resulted in activation.

Even the tight-binding inhibitor BILN 2061 produced activation. It was seen also at sub-nanomolar inhibitor concentrations, that is, lower than the enzyme concentration. VX 950, a slow binding mechanism-based inhibitor, showed no signs of activation when using 10 min preincubation, but activated when using 15 min. However, the activation was not enough to cancel out the time-dependent inhibition for this compound. Initial rates, that

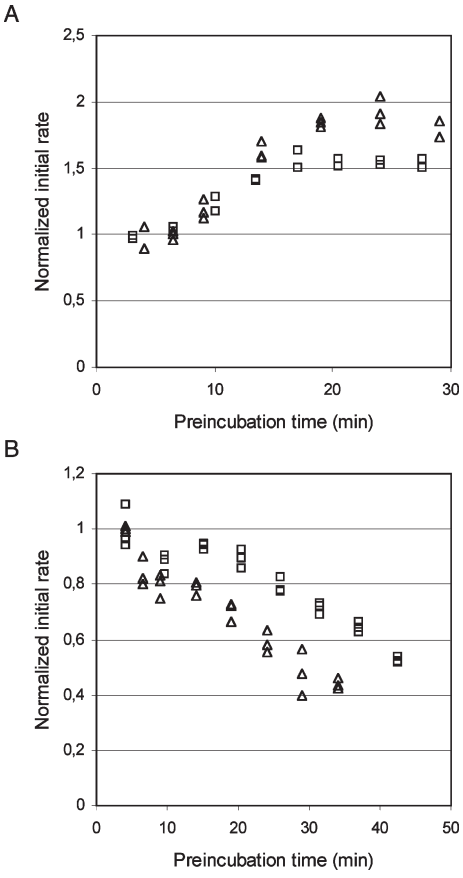


FIGURE 3: Effect of preincubation time using (A) 1 nM (□) or 2 nM (Δ) flNS3, 25 μM NS4A, 0.2 μM inhibitor **2**. Initial rates were normalized to activities for t = 3 min using 1 nM enzyme. (B) 2.5 nM (□) or 5.0 nM (Δ) VX-950 using 1 nM flNS3 and 25 μM NS4A. Initial rates were normalized to activities for t = 3 min using 2.5 nM inhibitor.

is, protease activity after 15 min preincubation, were lower than those obtained at 0 min, but higher than rates obtained directly before or after 15 min preincubation (Figure 3B). Because all but one of the studied inhibitors produced activation, the phenomenon is of a general nature not directly associated with inhibitor structure, mechanism, or potency, but rather with preincubation time.

Activation Primarily Affects k_{cat} and V_{max}(NS4A) and not K_m or K_d. The activation did not result in any deviations from classical Michaelis−Menten kinetics, only the rate of catalysis (k_{cat}). By including 0.2 μM inhibitor **2** (in the standard assay) k_{cat} was 1.01 ± 0.02 s^{-1}, while it was 0.83 ± 0.02 s^{-1} in the absence of the inhibitor. The corresponding K_m-values were 0.25 ± 0.025 μM and 0.21 ± 0.023 μM, respectively. Varying the NS4A concentration in the presence or absence of an inhibitor at activating concentrations (compound **2** at 0.2 μM) revealed that activation occurred over a range of different NS4A concentrations (Figure 4). Fitting the standard binding isotherm to the data resulted in a K_d of 3.8 ± 0.63 μM and a V_{max} of 0.72 ± 0.02 (V_{max} is without units since the initial rates were normalized) in the absence of the inhibitor, and K_d 5.2 ± 0.49 μM and V_{max} 1.18 ± 0.03 in its presence. These experiments show that the activation is an effect of increased protease activity (k_{cat} and V_{max}), and that it has no significant effect on the apparent affinity between flNS3 and substrate or NS4A (K_m and K_d).

Only the Assembled flNS3−NS4A Complex Is Activated by Inhibitors. The formation of active flNS3 was found to be very fast; the NS3-NS4A complex was formed already during the mixing procedure, preceding the start of measurements

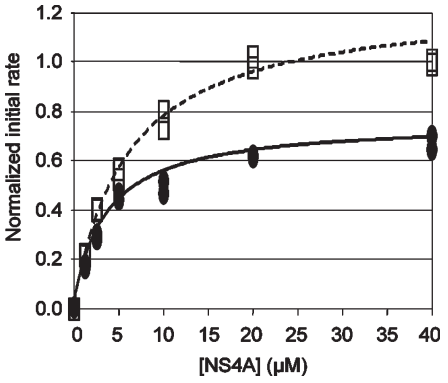


FIGURE 4: The effect of varying the NS4A concentration on the protease activity in the absence (●) (solid line) or presence (□) (dashed line) of 0.2 μM inhibitor **2** using 1 nM flNS3. Initial rates were normalized to when [NS4A] = 40 μM in the presence of the inhibitor. The lines represent the fitting of a standard binding isotherm to the two data sets, separately.

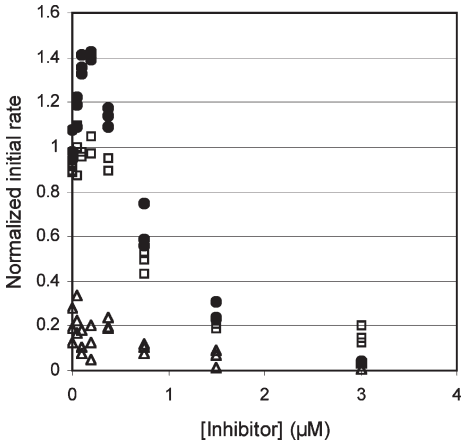


FIGURE 5: The effect of an activating inhibitor (inhibitor **2** at 0.2 μM) when preincubated with only 1 nM flNS3 (Δ), only 25 μM NS4A (□), or 1 nM NS3 and 25 μM NS4A (●) for 15 min prior to adding the missing component (i.e., NS4A or flNS3) and 0.5 μM substrate. Initial rates were normalized when flNS3 was preincubated with NS4A without inhibitor.

(data not shown). It was therefore possible to investigate how the inhibitor affected flNS3 in the absence of NS4A and how the inhibitor affected NS4A in the absence of flNS3. Preincubating inhibitor **2** with only flNS3 gave rise to just a small activation (Figure 5). However, this is interpreted to be a result of the activation of flNS3 with NS4A that had time to take place during the mixing procedure, rather than an effect of the inhibitor on free flNS3. Preincubating only flNS3 with the inhibitor for 15 min also resulted in over 80% loss of NS3 protease activity, indicating that NS4A is crucial for the stability of flNS3 and that the inhibitor was not able to fill this role at the concentration tested.

Similarly, a small activation was seen when only NS4A was preincubated with the inhibitor prior to adding flNS3 and substrate (Figure 5). This activation is also believed to have taken place during the mixing rather than as a result of any effect the inhibitor had on NS4A. Furthermore, adding NS3 to preincubated NS4A did not result in a significant loss of protease activity, most probably due to an effect of keeping NS3 at a higher concentration (about 85 nM) and at room temperature, rather than 30 °C, prior to adding it to NS4A.

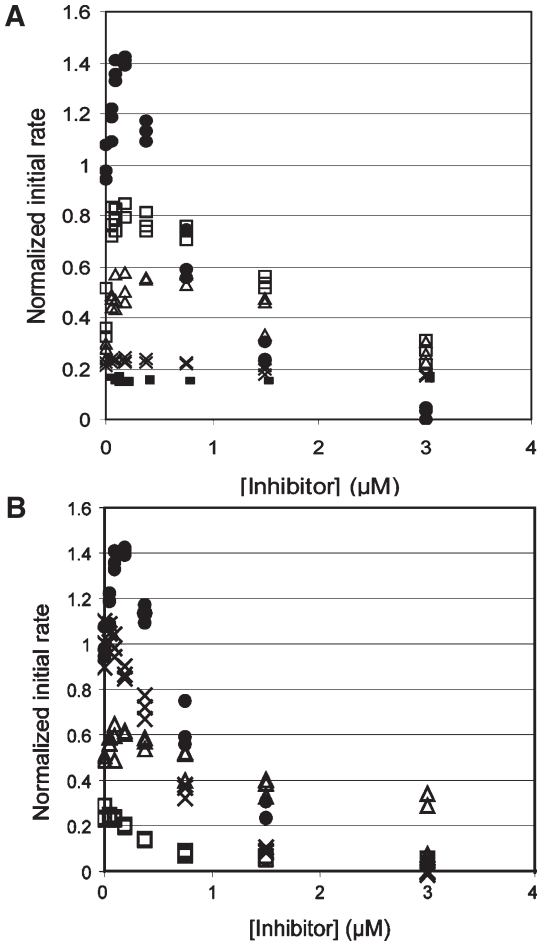


FIGURE 6: Effects of modified assay conditions on activation using 0.2 μM inhibitor **2**, 1 nM flNS3 and 25 μM NS4A with (A) 0 mM (●), 150 mM (□), 300 mM (Δ), 600 mM (×), or 1 M (-) NaCl. Initial rates were normalized to when [I] = 0 and [NaCl] = 0. (B) The standard buffer (●), a buffer with 0.1% CHAPS instead of 0.1% *n*-octyl-β-D-glycopyranoside (×), a buffer containing no glycerol (Δ) or a buffer with pH 6.0 (□). Initial rates were normalized to when [I] = 0 using the standard buffer.

Effect of Assay Conditions on the Activation. The activation was reduced by increased ionic strength, but did not completely disappear until 1 M NaCl was included in the buffer, at which time the activity of flNS3 was reduced by over 80% (Figure 6A). Similarly, exclusion of glycerol from the buffer resulted in a 50% loss of protease activity and a reduction in the activation (Figure 6B). When using a buffer with pH 6.0 instead of 7.4, the protease activity of NS3 was reduced by over 75%, even though the degree of activation was reduced. However, using CHAPS instead of *n*-octyl-β-D-glycopyranoside greatly reduced the activation without affecting NS3 protease activity. Still, taken together, the activation was not readily disrupted by changes in ionic strength, pH, or glycerol concentration, indicating that the phenomenon is rather robust.

Truncated NS3/NS4A Is Less Active than flNS3 and Not Activated by Inhibitors. As a control, the commonly used truncated NS3 from subtype 1b was used. It consisted of the protease domain with the short peptide corresponding to the NS4A cofactor fused to its N-terminus (note that the NS4A is attached to the C-terminus of the NS3 protease in the native enzyme). It was almost inactive in the assay buffer used for full-length NS3. By varying the buffer composition, it was found that the highest activity was obtained when using the same buffer as

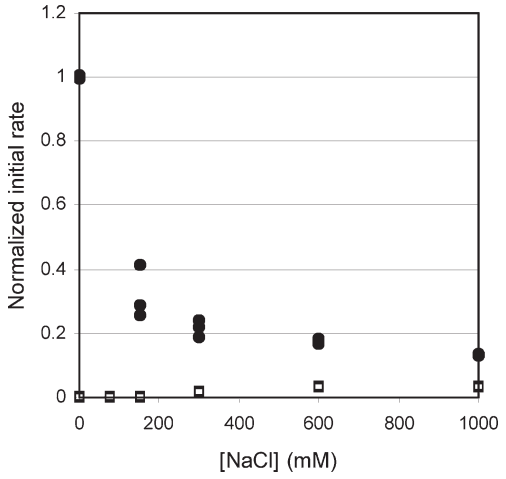


FIGURE 7: The effect of different ionic strengths on protease activity of 1 nM flNS3 with 25 μM NS4A (●) or 1 nM truncated NS3/NS4A (□) using 0.5 μM substrate concentration. Initial rates were normalized to when [I] = 0 using flNS3.

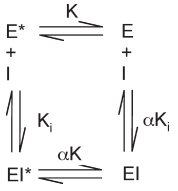
for full-length NS3, supplemented with 1 M NaCl. However, truncated NS3/NS4A was still much less active than full-length NS3; at 1 M NaCl it had less than 5% of the activity full-length NS3 had at optimal conditions (Figure 7).

As a consequence, direct comparisons between the two enzymes proved difficult. An assay buffer supplemented with 600 mM NaCl was used as a compromise where flNS3 was still active and activated by the inhibitor, and where NS3/NS4A also showed measurable protease activity. The K_m for NS3/NS4A was 2.2 ± 0.3 μM under these conditions (data not shown), which is 10 times higher than for flNS3 in a buffer without NaCl. Adding extra NS4A to NS3/NS4A had no effect on protease activity or activation, as expected considering the covalently attached NS4A in this construct. Using the buffer with 600 mM NaCl or other buffers without glycerol, low pH, or CHAPS failed to produce any activation (data not shown). Since the K_m was 10-fold higher in the buffer used, use of a higher substrate concentration, resulting in the same K_m/[S] ratio as for flNS3, was tested but did not result in any activation. Increasing the enzyme concentration did not produce any activation either. Thus, the inhibitor could not be seen to activate this form of truncated NS3 protease under any of the tested conditions.

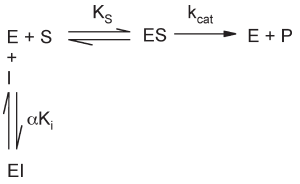
Proposed Activation Mechanism. In order to define a mechanism for the activation, various models were investigated. A model involving an enzyme memory process was identified to describe the phenomenon well (see Discussion). Several assumptions were made in order to keep the model as simple as possible. First, all of the NS3 participating in the mechanism was assumed to be in the form of NS3-NS4A complexes. This is conceivable since an NS4A concentration higher than the apparent equilibrium dissociation constant for the NS3-NS4A complex was used and any free NS3 would not contribute to the measured activity since NS3 not bound to NS4A is practically inactive. Second, two enzyme species were assumed to be present, E* and E. Both can bind the inhibitor, but with different affinities, and only E can process substrate. The partition of the two free enzyme species is defined by the equilibrium constant K.

Upon the addition of the inhibitor and during the preincubation time, the system equilibrates according to the differential affinity of the inhibitor for each of the two enzyme conformers, as depicted in Scheme 1:

Scheme 1



Scheme 2



The coefficient α is a measure of the ratio of affinities of the inhibitor for the two enzyme forms.

When substrate is added, only E can process it to product. It was assumed that the measurement of initial velocity is faster than the re-equilibration of the enzyme conformers, whether free or inhibited. Thus, during the measurement there is no significant net conversion between the conformers E* and E and just the enzyme in the "E branch" (E + EI) of Scheme 1 is involved in catalysis (Scheme 2):

If the substrate concentration is of the order, or higher than, the K_m, the inhibitor is quickly displaced. In these circumstances, activation is seen if the inhibitor has a higher affinity for E than for E* (α < 1), as the "E branch" contains more enzyme than the control without inhibitor. For this mechanistic model, eq 1 was derived (see Supporting Information):

$$v_o^i = \frac{k_{cat} \times [e] \times [S]_o}{\left(K_m \times \left(1 + \frac{[I]_o}{\alpha \times K_i}\right) + [S]_o\right) \times (K \times \beta + 1)} \quad (1)$$

where β is

$$\beta = \frac{\left(1 + \frac{[I]_o}{K_i}\right)}{\left(1 + \frac{[I]_o}{\alpha \times K_i}\right)} \quad (2)$$

and [e] is the total concentration of enzyme in the assay ([E] + [E*]).

For $[I]_o = 0$, eq 1 results in the expression for the control of enzyme activity:

$$v_o = \frac{k_{cat} \times [e] \times [S]_o}{(K + 1) \times (K_m + [S]_o)} \quad (3)$$

The standard equation for competitive inhibition (eq 4) was used as a reference:

$$v_o = \frac{k_{cat} \times [e] \times [S]_o}{K_m \times \left(1 + \frac{[I]_o}{K_i}\right) + [S]_o} \quad (4)$$

Both eqs 1 and 4 were used for nonlinear regression analysis of the experimental data and the estimation of K_i-values for the inhibitors, Figure 8 and Table 1. VX 950 was omitted from the analysis due to its time-dependent inhibition. The value of K was determined to be around 100. The resulting fits to the data were generally better when using eq 1 than when using eq 4, most clearly seen at low inhibitor concentrations (insets, Figure 8).

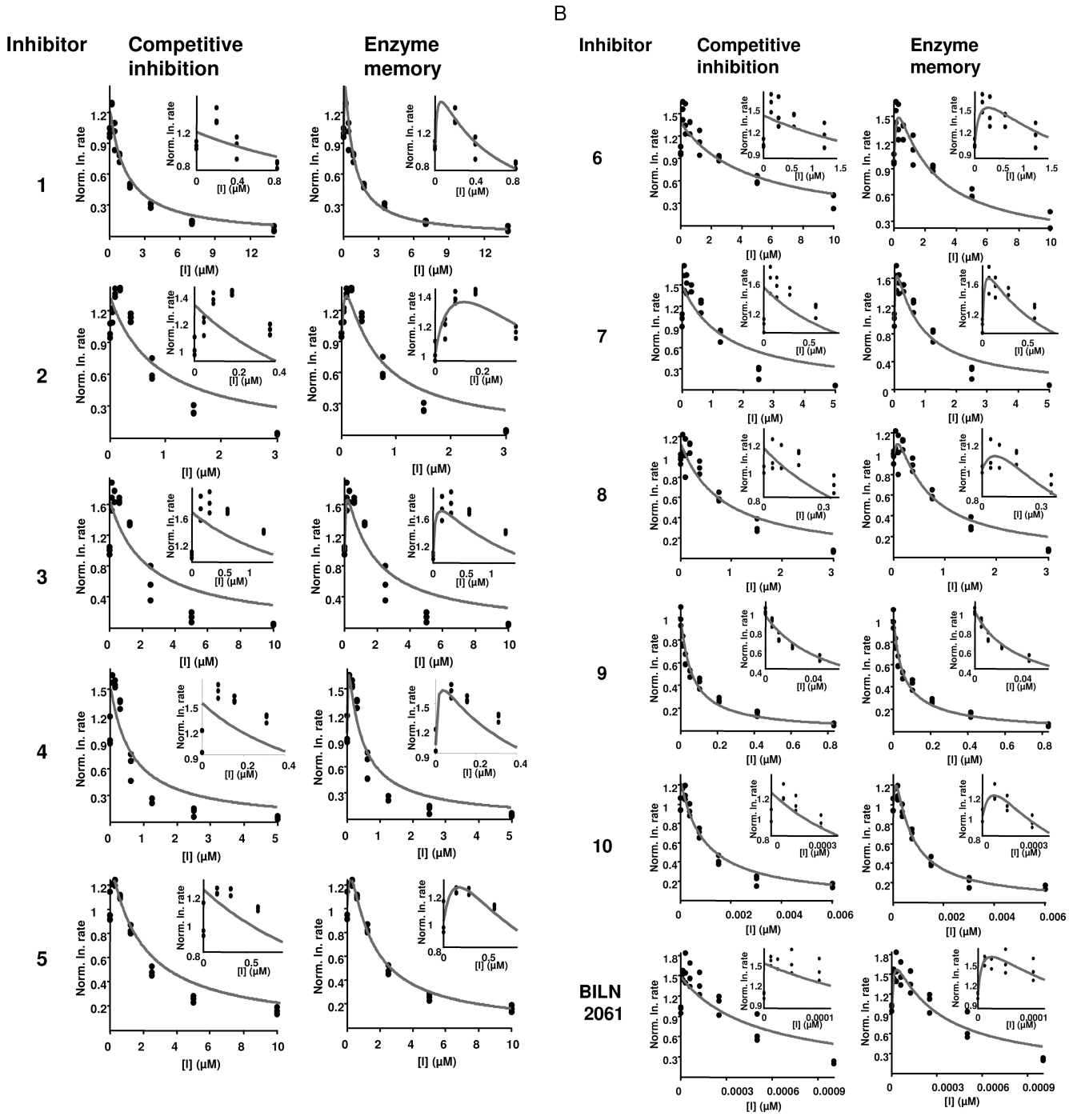


FIGURE 8: Nonlinear regression analysis of models describing a simple competitive inhibition (eq 4) or an extended inhibition mechanism including a second species of the enzyme (eq 1). Initial rates were normalized to when [I] = 0. The corresponding kinetic parameters are presented in Table 1. Insets show an expansion of the low concentration region.

It was found that α was below 1 for all activating compounds (Table 1). Moreover, the higher the activation, the lower the value of α. For example, compounds **8** and **10** with around 20−30% activation displayed higher values of α than compounds **1** and **7** which had around 50−60% activation.

Limitations in the model were observed for compounds **3** or **4** for which it was not possible to determine any reasonable values for α or K_i. Comparisons of the values obtained using eq 1 and 4 only showed up to a 2-fold difference in K_i-values, but the standard errors were reduced when using eq 1 (Table 1). An exception was inhibitor **9**, for which eq 1 did not fit better than eq 4. This is expected since inhibitor **9** was not activating, reflected in its value of α being over 1. Also for BILN 2061, the fit was best with eq 4. However, this inhibitor and inhibitor **10** are both tight-binding inhibitors, something that is not accounted for in either eq 1 or eq 4.

DISCUSSION

Excluded Mechanistic Explanations for Activation by Inhibitors. After the possibility was discarded that the activation of NS3 protease by low concentrations of inhibitors was due to some nonenzymatic artifact, different mechanistic explanation models were sought. Protection against autoproteolysis was excluded as an explanation since less than 1% of NS3 protease

Table 1: K_i-Values for NS3 Protease Inhibitors Determined with a Model Accounting for Inhibitor Activation via an Enzyme Memory Mechanism (eq 1) and a Standard Equation for Competitive Inhibition (eq 4)[a]

	competitive inhibition	enzyme memory	
inhibitor	K_i (nM)	K_i (nM)	α
1	330 ± 70	740 ± 110	0.026
2	180 ± 58	470 ± 64	0.055
3*	520 ± 200	n.d.	n.d.
4*	150 ± 53	n.d.	n.d.
5	500 ± 100	1000 ± 45	0.092
6	1100 ± 260	3200 ± 390	0.020
7	290 ± 100	1600 ± 440	0.011
8	170 ± 33	380 ± 52	0.203
9	15 ± 1.5	39 ± 15	2.5
10	0.22 ± 0.034	0.45 ± 0.022	0.119
BILN 2061	0.12 ± 0.036	0.58 ± 0.43	0.082

[a]The degree of activation is defined by α. All determinations were made at least in triplicates and the standard deviations from the nonlinear regression analysis are given. *These inhibitors had an activation higher than 60%, and the kinetic parameters could not be determined accurately using eq 1.

activity was lost during the time frame used for these experiments and a stabilizing effect would not increase the protease activity, only reduce the rate of inactivation. Moreover, the time dependency for activation was not consistent with a mechanism involving stabilization of an autoproteolytic enzyme, and one inhibitor did not activate the enzyme although it would be expected to reduce the degree of autoproteolysis as well as the other inhibitors.

Activation could potentially arise as an indirect cooperative or allosteric effect where inhibitor binding to an adjacent enzyme molecule or an allosteric site in the same molecule affects the enzyme activity. Such effects are not unconceivable, considering that long-range interactions affect NS3. For example, oligomerization of NS3 in solution increases the helicase activity (*32*, *33*), and interactions between NS3 and other HCV proteins modulate its helicase activity (*14*, *34*, *35*). Moreover, intramolecular interactions between the two domains of NS3 have been described (*14*, *16*−*18*) as well as stabilization of NS3 in the absence of NS4A by substrate and inhibitors (*36*−*38*). However, no signs of cooperativity were seen in the substrate saturation curves and no second site for protease inhibitors, which could confer allosteric regulation, has been reported to exist in NS3. Nevertheless, the current experimental setup cannot fully exclude this possibility.

Activation via an Enzyme Mnemonic Mechanism. The plausible explanation is that of an enzyme memory, or single-site cooperativity, model. It can be a result of a stabilizing effect of a ligand on one of two enzyme forms, shifting the equilibrium toward one of the species (*30*, *39*). Enzymes that function in this way are also known as mnemonic enzymes. The mechanism is also a form of enzyme hysteresis, a phenomenon that has only been established for a few enzymes (*31*, *40*−*43*). An enzyme memory process displays no deviation from simple Michaelis−Menten kinetics in the substrate binding isotherms (*30*) and is only observed for compounds fulfilling special structural and kinetic properties. This matches the current observations for HCV NS3 and is consequently the most likely, or least unlikely, reason for the activation.

The activation observed here required that the enzyme was preincubated with the inhibitor for several minutes, which is consistent with a slow isomerization of the enzyme. The fact that BILN 2061 activated the enzyme at inhibitor concentrations below the enzyme concentration also suggests an effect involving lower than stoichiometric amounts of the inhibitor, excluding several of the alternative explanations. The derived equation (eq 1) for this mechanism described the activation data better than the standard equation (eq 4), except for inhibitors displaying very high activation levels. A more complex model, without the simplifications assumed for derivation of eq 1, could possibly result in a better fit but would require more extensive data. Still, the present model and equations represent a basic framework for the activation mechanism. It describes the experimental data well and is consistent with the conformational features and dynamics of the enzyme. Still, the relationship between the enzyme concentration and the inhibitor identity with a varying preincubation time to reach maximal activation are not easily explained by the simple mnemonic model used here.

Structure−*activity Relationships and Effects of Assay Conditions*. A structure−activity relationship for the activation could not be elucidated since inhibitors varying in size, polarity, mechanism, or potency all activated NS3. The only difference between the nonactivating compound (inhibitor **9**) and several activating compounds (e.g., **6**, **8**, and **10**) was that it had a shorter C-terminus. Although it can be speculated that a certain length on the C-terminal side is needed for activation, the structural basis for inhibitors that produce activation remains to be investigated. The degree of activation was different for the various compounds, but since the effect of time was not investigated for all of them, it could simply be that maximal activation was reached at different times for different inhibitors.

In order to elucidate the nature of the activation mechanisms, the effects of various assay conditions were studied. Despite the difficulties in interpreting such effects in multicomponent systems, modifications influenced the protease activity of NS3 more than its activation by inhibitors. The only exception was when switching detergent from CHAPS to *n*-octyl-β-D-glycopyranoside, which resulted in reduced activation and retained protease activity. The reason for this is not clear, but the different nature of these detergents could provide important clues to the forces behind the activation phenomenon.

Molecular Basis for Enzyme Activation. Two different explanations for the observed activation have been found to be consistent with the experimental data and the mechanistic model. The first is based on the initial hypothesis that the helicase domain would be involved since, in contrast to full-length NS3, the protease of the truncated NS3 was not activated by any inhibitor under any assay condition tested. If the helicase domain in full-length NS3 is in close proximity to the protease domain, the binding of a protease inhibitor to the active site could possibly slowly induce a conformational change, resulting in protease activation. The exact nature of this presumed conformational change is not known. By comparing the data obtained here with previous data from inhibition studies of full-length NS3 variants containing amino acid substitutions around the active site (R155Q, A156T, and D168V (*26*) and Q526A, H528A, H528S (*16*)), it was found that all these enzymes were all activated to a similar degree as the wild type (not shown). Thus, the activation is unaffected by these amino acids changes, which further supports the robustness for this phenomenon.

A second explanation involves a mechanism where the inhibitor only influences the active site. This may also be different for the two different forms of the enzyme since the protease domain of the truncated NS3 used in this experiment had the

central part of NS4A covalently attached to its N-terminus, whereas NS4A was added as a free peptide prior to the measurements with full-length NS3. Thus, activation could be due to a suboptimal complex formation between full-length NS3 and NS4A or of the alignment of the catalytic triad, which is remedied by using a low concentration of a protease inhibitor. There is evidence for this since NS3 is more active if coexpressed with NS4A than if NS4A is supplied as a free peptide just prior to protease activity measurements (*17*). The absolute requirement for preincubation to see activation also supports this hypothesis and could thus be similar to the activation of NS3 by substrate and inhibitors in the absence of NS4A (*36*−*38*). However, this form of activation or stabilization was mainly attributed to prime-site residues in the substrate and the inhibitors (*38*, *44*), a structural feature that is not present in the inhibitors studied here.

The perfect enzyme controls for these experiments have unfortunately not been available since we have been unsuccessful in purifying enzyme from the reference constructs made and expressed for this purpose. Specifically, production of the full-length NS3 without the extended affinity tag in the N-terminus has not been possible since the low amounts of expressed protein could not be purified without this affinity tag (*20*). It can therefore not be excluded that the tag interferes with the protease activity of the flNS3 used. Similarly, attempts to purify the truncated version of NS3 (with or without the tag) have also been unsuccessful (data not shown). The commercially available truncated NS3 was therefore used to determine the effects of the helicase domain. Unexpectedly, it was found that the truncated NS3/NS4A was significantly less active than the full-length NS3, under all explored assay conditions. This was surprising since truncated NS3/NS4A has been shown to be active in various buffer conditions and readily comparable to flNS3 (*7*, *45*, *46*). This difference is not believed to be due to differences in genotypes (1a and 1b) or the fused NS4A to the N-terminus, since these variations in sequence or modifications have been shown to be insignificant (*7*, *45*, *47*, *48*). Thus, even with these differences, if NS4A has fully activated the truncated enzyme in this particular construct, why does it display so much lower activity compared to the full-length NS3? Although not all possible assay conditions were tested in this study, the truncated protease never came close to the activity of full-length NS3 under any of the explored conditions. This observation is yet another reason for not assuming that the protease domain alone is a good model system for the NS3 protease functionality (see below).

The current study cannot distinguish between the above two mechanisms for the activation, but the proposed model and derived equations are valid in both cases. In order to fully explore this enzyme memory process and to understand the underlying process, usage of other enzyme constructs and methodologies have to be implemented.

Evaluating Potencies of Activating Inhibitors. Although the activation is an interesting phenomenon from an enzymological point of view, in practice, the activation phenomenon has only a small effect on the estimation of K_i-values for inhibitors−the simple aim of many studies of NS3 protease. It is also possible to tune the assay to avoid the activation all together by using short preincubation times or by using CHAPS as detergent. However, it should be noted that since the used equations describe quite different mechanisms, a simple comparison between the K_i-values is not easily done. Still, the derived equation for activating inhibitors is more accurate for describing the mechanism when activation occurs and is expected to provide additional information (α-values) about studied inhibitors. It remains to be determined how significant this phenomenon for in vivo conditions is and what the ideal characteristics of inhibitors are.

Model Systems for Evaluation of NS3 Protease Inhibitors. Given the immense number of compounds that have been tested as inhibitors toward HCV NS3, it is strange that this activation phenomenon has not been reported before. Since certain criteria for both inhibitor structure and assay conditions have to be fulfilled in order to produce activation, it can obviously be overlooked. Activation is apparently only seen when using the full-length NS3 enzyme and not truncated NS3 protease, which is most commonly used for enzymatic studies of NS3. Two recent studies have provided conflicting conclusions on the suitability of truncated NS3 as a model system for evaluation of inhibitors, in comparison to full-length enzyme (*17*, *46*). In our hands, truncated NS3 was not as active as full-length enzyme, nor was it activated by any of the studied inhibitors. Even with the variations in sequence and construct, the difference in protease activity should not be this large unless there are other major factors involved.

Our conclusion is that even though it may be possible to find assay conditions where truncated and full-length NS3 are equally active, it does not mean that they are equal. It is possible that truncated NS3 is just as good a model system as full-length NS3 for drug discovery efforts, for example, lead identification. However, full-length NS3 is the in vivo form of the enzyme and using truncated NS3 for enzymatic studies can result in incorrect conclusions or assumptions about the characteristics, function, or regulation of the protein.

Although full-length NS3 complemented with NS4A peptide in solution can be argued to be more physiologically relevant than truncated NS3 protease, it is also an artificial compromise. The native enzyme is associated with a membrane and in complex with not only full-length NS4A but possibly also with other components of the replication complex or various host factors. Since membrane association is believed to greatly restrict the enzyme flexibility (*19*), an activity assay representing this system is needed to further increase our understanding of the protein dynamics taking place.

CONCLUSION

An enzyme memory model accurately described the activation of full-length NS3 protease by low concentrations of inhibitors. This model, as well as the derived equations based on it, is useful for understanding and describing similar activation phenomena. The exact mechanism behind the activation of NS3 remains to be investigated, but the current study sheds light on an obscure event that can occur when measuring enzyme inhibition.

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SUPPORTING INFORMATION AVAILABLE

The definition of the enzyme memory model for activation of HCV NS3 protease by inhibitors and the corresponding equations. This material is available free of charge via the Internet at http://pubs.acs.org.

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