The effect of prime-site occupancy on the hepatitis C virus NS3 protease structure

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Abstract

We recently reported a new class of inhibitors of the chymotrypsin-like serine protease NS3 of the hepatitis C virus. These inhibitors exploit the binding potential of the S′ site of the protease, which is not generally used by the natural substrates. The effect of prime-site occupancy was analyzed by circular dichroism spectroscopy and limited proteolysis-mass spectrometry. Generally, nonprime inhibitors cause a structural change in NS3. Binding in the S′ site produces additional conformational changes with different binding modes, even in the case of the NS3/4A cofactor complex. Notably, inhibitor binding either in the S or S′ site also has profound effects on the stabilization of the protease. In addition, the stabilization propagates to regions not in direct contact with the inhibitor. In particular, the N-terminal region, which according to structural studies is endowed with low structural stability and is not stabilized by nonprime inhibitors, was now fully protected from proteolytic degradation. From the perspective of drug design, P-P′ inhibitors take advantage of binding pockets, which are not exploited by the natural HCV substrates; hence, they are an entry point for a novel class of NS3/4A inhibitors. Here we show that binding of each inhibitor is associated with a specific structural rearrangement. The development of a range of inhibitors belonging to different classes and an understanding of their interactions with the protease are required to address the issue of the most likely outcome of viral protease inhibitor therapy, that is, viral resistance.

Keywords: Hepatitis C virus NS3 serine protease; near-UV circular dichroism; limited proteolysis-mass spectrometry; protease inhibitors; conformational changes

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Abbreviations: CD, circular dichroism; CHAPS, 3-[(cholamidopropyl) dimethylammonio]-1-propansulfonate; DIEA, diisopropyl-ethylamine; ESMS, electro-spray mass spectrometry; Fmoc, 9-fluorenylmethyloxycarbonyl; HCV, hepatitis C virus; HOBt, *N*-hydroxybenzotriazole; PyBOP, (benzotriazol-1-yloxy)tris(pyrrolidino)phosphonium hexafluorophosphate; *t*-Bu, *tert*-butyl; TFA, trifluoroacetic acid.

We follow the nomenclature of Schechter and Berger (1967) in designating the cleavage sites as P6-P5-P4-P3-P2-P1 . . . P1′-P2′-P3′-P4′, etc., with the scissile bond between P1 and P1′ and the C terminus of the substrate on the prime site. The binding sites on the enzyme corresponding to residues P6-P5-P4-P3-P2-P1 . . . P1′-P2′-P3′-P4′ are indicated as S6-S5-S4-S3-S2-S1 . . . S1′-S2′-S3′-S4′, etc.

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The hepatitis C virus causes a predominantly chronic viral infection that affects ~3% of the world population, for which existing antiviral treatments present problems of toxicity and limited effectiveness. A key role in the maturation of the hepatitis C virus is due to the viral serine protease that is encoded in the N-terminal domain of the multifunctional NS3 protein. Therefore, the NS3 protein is one of the most important targets for drug development against HCV. Although NS3 shows proteolytic activity of its own, the efficient processing of all the cleavage sites, necessary for viral maturation, is strictly dependent on the formation of a fully competent proteolytic complex between NS3 and another viral protein, NS4A.

Structural studies (Kim et al. 1996; Love et al. 1996; Yan et al. 1998; Barbato et al. 1999; Yao et al. 1999), as well as spectroscopic (Bianchi et al. 1997; Orrù et al. 1999) and

kinetic analysis (Landro et al. 1997), have highlighted that binding of the cofactor NS4A causes a structural rearrangement of NS3, which results in a fully proteolytically competent enzyme. Complex formation has a key role in the stabilization of the N-terminal domain of the enzyme. This region comprises the S′ site, which is part of the substrate binding site and it contains residues of the catalytic machinery. The substrate binding region is shallow and entirely solvent exposed and as a consequence the enzyme requires at least a decapeptide substrate (Steinkühler et al. 1996; Urbani et al. 1997). The design of inhibitors is therefore particularly challenging.

This notwithstanding, several potent peptide inhibitors of the NS3/4A protease have been described, essentially based on the N-terminal cleavage product (Ingallinella et al. 1998; Llinàs-Brunet et al. 1998). This class of competitive inhibitors takes advantage of binding in the S subsite of the enzyme in a well-defined extended conformation as observed for other proteolytic enzymes (Rich 1990; Bianchi et al. 1999; Cicero et al. 1999; LaPlante et al. 1999a; Barbato et al. 2000). In particular, circular dichroism spectroscopy and the joint use of limited proteolysis and mass spectroscopy showed that product-based inhibitors bind according to an induced-fit mechanism (Bianchi et al. 1999). Different binding modes could be achieved in the absence of cofactor, whereas in the presence of cofactor, all inhibitors showed the same binding mode to NS3, with a small rearrangement of protease tertiary structure. Cofactor binding induces a NS3/4A conformation that is already, but not entirely, preorganized for substrate binding, affecting the S′ site, as well as the S site. Conversely, occupancy of the substrate binding site by the product-based inhibitor induces an overall stabilization of the protease complex, also influencing the region involved in cofactor binding. Accordingly, recent NMR solution studies of NS3/P inhibitor complexes suggested that the proteolytic competent state of the NS3 protease is only achieved through complexation of both the cofactor and the substrate (Barbato et al. 2000).

At variance with the P region, very little is known about the interaction of the P′ region of the substrate with the S′ region of the enzyme. Enzymological studies (Landro et al. 1997; Urbani et al. 1997; Zhang et al. 1997) highlighted that, differently from the P region, the P′ region of the substrate is important for catalysis although it contributes very poorly to ground-state binding to the enzyme. Accordingly, peptides derived from the P′ region of the substrates do not bind to the enzyme. Despite that, on the basis of the structural information available, the S′ subsite of the NS3/4A shows the presence of pockets whose binding potential was exploited by us, leading to the discovery of a novel class of substrate-derived peptide inhibitors (Ingallinella et al. 2000). These competitive inhibitors span from P6 to P4′, therefore also taking advantage of binding in the S′ site of the enzyme. In this study this series of inhibitors was selected as a structural probe to gain insight into the binding mechanism in the S′ region of the NS3 protease domain from the HCV J strain. As for product-based inhibitors (Bianchi et al. 1999), we used circular dichroism spectroscopy and the combination of limited proteolysis and mass spectrometry to study the substrate binding site and the effect of inhibitor binding on the protease tertiary structure.

Results

Inhibitors used in this study

In our previous work (Ingallinella et al. 2000) we reported a series of peptide inhibitors of the NS3 protease, which comprise both the P and the P′ regions. The decapeptide inhibitors used in this study span from P6 to P4′. Their sequences and inhibitory potencies are reported in Table 1. These are noncleavable peptides incorporating chemical changes of the putative scissile bond P1-P1′ in order to prevent proteolysis by NS3. The P1′ residue consists of a secondary amino acid such as proline, as in PepB and PepC. The proline residue introduces a distortion of the putative cleavage site, thus impairing cleavage as suggested by Landro et al. (1997). On the other hand, the inhibitor PepD contains a suboptimal P1 residue and a methyl group on the N-amide between P1 and P1′ (Ingallinella et al. 2000). All three peptides share the same sequence in the P region obtained by investigation by combinatorial chemistry of hexapeptide product inhibitors binding to NS3 (Ingallinella et al. 1998). PepD and PepB are decapeptides with the residues of the P′ region based on the natural viral cleavage sites 4A/B and 5A/B. In particular the P′ region of PepD is based on the 4B N-terminal region Ala-Ser-His-Leu. In PepB the P′ sequence Pro-Ser-Nle-Leu resembles the 5A/B sequence with the isosteric substitution of the methionine for norleucine, the replacement of the aromatic Tyr with the 4B Leu residue, and the Proline at the P1′ position. Also PepC shows the same sequence homology in positions P6-P1′ as for PepB, but in this peptide, P2′ and P3′ are replaced with

Table 1. *NS3-inhibitors spanning P6–P1 and P6–P4′ analyzed in this study by circular dichroism (CD) spectroscopy and limited proteolysis mass spectrometry (MS)*

Peptide	Sequence[a]	IC_{50} (nM)[b]
A	Asp-glu-Leu-Ile-Cha-Cys-OH	15
B	Asp-glu-Leu-Ile-Cha-Cys-Pro-Nle-Ser-Leu	10
C	Asp-glu-Leu-Ile-Cha-Cys-Pro-Cha-Asp-Leu	<0.2
D	Asp-glu-Leu-Ile-Cha-Abu-(Me)Ala-Ser-His-Leu	5000

[a] Abbreviations: Cha, β-cyclohexylalanine; glu, D-glutamic acid; Nle, Norleucine. N-Methylation of the amide nitrogen is indicated with an (Me) before the three letter code of the amino acid.
[b] IC_{50} values for NS3/Pep4A protease were determined as described in Ingallinella et al. 1998. Under our experimental conditions, K_i~0.5 IC_{50}

residues obtained by combinatorial chemistry studies of the P′ region in the context of decapeptide sequences (Ingallinella et al. 2000). As indicated in Table 1, PepB and PepC show >100-fold difference in their inhibitory potencies. This difference reflects the contribution in binding of the optimized P2′ and P3′. To compare the results of this study with those previously obtained on P-based inhibitors (Bianchi et al. 1999) and to evaluate the contribution of the P′ region, the hexapeptide PepA was used as a control peptide spanning P6-P1 and with the same sequence in the P-region. In the P-based inhibitors the C-terminal carboxylate of the P1 residue is one of the main contributors to the binding energy, a contact which lacks in the P-P′ inhibitors.

Limited proteolysis-mass spectrometry

The limited proteolysis-mass spectrometry technique is a useful tool for the structural analysis of proteins (Zappacosta et al. 1996). This approach is based on the evidence that exposed, weakly structured, and flexible regions of a protein can be recognized by a proteolytic enzyme. Here we employed the limited proteolysis by chymotrypsin to investigate the interaction of different hexa- and decapeptide inhibitors with the NS3 protease. The experiments were carried out on the binary and ternary complexes in which each inhibitor was incubated with the isolated NS3 or the NS3/Pep4A heterodimer, respectively. The results were compared with those previously obtained on NS3 (Bianchi et al. 1999; Orrù et al. 1999; Urbani et al. 1999). The differences in the proteolytic patterns were analyzed to identify the protein regions involved in the molecular interactions (Scaloni et al. 1998; Atkinson et al. 2000). Particularly, the specific conformational changes induced in the three-dimensional structure of NS3 by the differently optimized P and P′ regions of the inhibitors were studied.

In all experiments, formation of the complexes was performed by incubating NS3 or the NS3/Pep4A heterodimer with a 10:1 molar excess of individual peptide inhibitors at pH 7.5 and 25°C for 15 min prior to the proteolytic enzyme addition. Chymotrypsin was selected as conformational probe because previous experiments had shown that this enzyme was able to provide structural information on different regions of NS3 (Bianchi et al. 1999; Orrù et al. 1999; Urbani et al. 1999). The extent of the enzymatic hydrolyses was monitored on a time-course basis by sampling the incubation mixtures at appropriate time intervals, followed by high performance liquid chromatography (HPLC) fractionation of the fragments generated. Individual chromatographic peaks were manually collected and identified by ESMS, leading to the assignment of the preferential cleavage sites.

Figure 1 shows the ribbon representation of the crystal structure of the NS3/Pep4A complex (Yan et al. 1998). The backbone of the decapeptide inhibitor PepC has been modeled by energy minimization and molecular dynamics (Ingallinella et al. 2000). In this figure, the chymotrypsin sensitive sites in the NS3 structure have been highlighted.

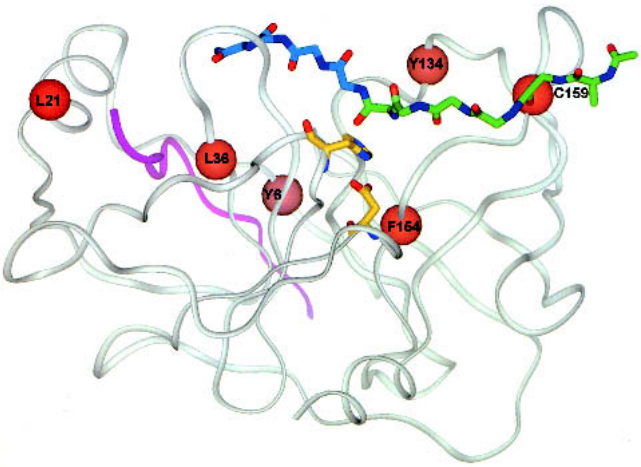

Fig. 1. Ribbon representation of the backbone of the crystal structure of the NS3 protease/Pep4A complex (Yan et al. 1998). A gray ribbon is used for the backbone of the NS3 protease and a magenta ribbon for Pep4A. The protease residues of the catalytic triad are represented in yellow sticks. The backbone of a decapeptide inhibitor was modeled by energy minimization and molecular dynamics (Ingallinella et al. 2000). The peptide is shown as a stick presentation, with the P region in green and the P′ in blue. The red spheres indicate the chymotrypsin sensitive proteolytic sites in the NS3 structure.

First the topology of the binary complexes between NS3 and the two decapeptide inhibitors B and C was analyzed. Figure 2 shows the HPLC profiles of the aliquots withdrawn following 60 min of chymotrypsin incubation of the complexes NS3/PepB (Fig. 2A) and NS3/PepC (Fig. 2B). When these results were compared with those obtained on the isolated NS3 protease reported in Table 2 (Orrù et al. 1999), a number of considerations could be drawn. A higher chymotrypsin to NS3 substrate ratio was needed in all experiments with the inhibitors (from 1:150 for the NS3 to 1:100 for the inhibitor complexes), suggesting that the binding of inhibitors increased the overall compactness of the NS3 protease. The distribution of the preferential proteolytic sites observed in the presence of PepB (Fig. 2A) was very similar to that displayed by the isolated protein (Table 2). In particular the preferential chymotryptic sites were observed at positions Tyr 6, Leu 21, and Leu 36, as indicated by the rapid appearance of the two complementary peptide fragments 2–6 and 7–186 (peaks 1 and 10 in Fig. 2A) and of fragments 7–21, 22–186, 22–36, 37–186, (peaks 7, 2, 8, and 9 in Fig. 2A, respectively). All these sites are located in the NS3 N-terminal domain, a region that is directly involved in the cofactor NS4A binding. Further cleavages were observed in the substrate binding site, within the C-terminal domain of the NS3 protease, at Tyr 134, Phe 154, and Cys 159, highlighted by the peptide fragments 135–154, 155–

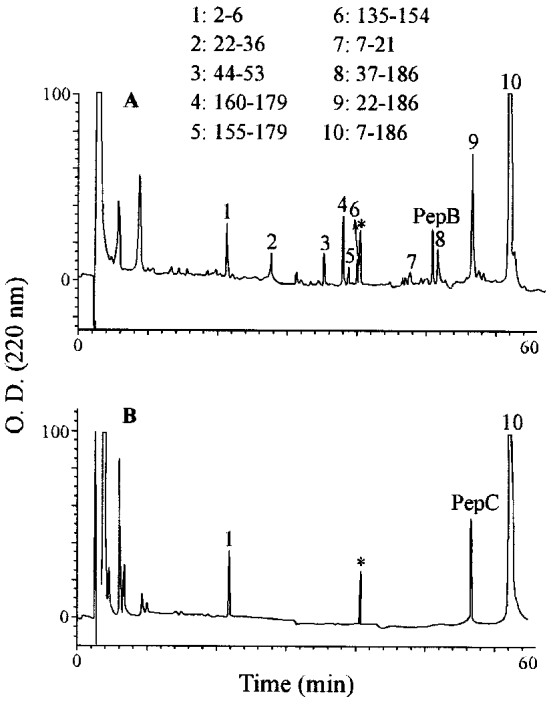

Fig. 2. Reverse-phase HPLC analysis of NS3/PepB (*A*) and NS3/PepC (*B*) complexes digested with chymotrypsin under controlled conditions using an enzyme/substrate ratio of 1:100 (w/w) in the presence of a 1:10 molar excess of each peptide. Individual fractions were collected and analyzed by ESMS. The CHAPS peak is marked with an asterisk; peaks corresponding to the inhibitor PepB and PepC are also indicated.

179, and 160–179 (peaks 6, 5, and 4 in Fig. 2A, respectively).

Conversely, a complete protection of most of the preferential proteolytic sites was observed for the NS3/PepC complex (Fig. 2B) with no evidence of processing at Leu 21, Leu 36, and Tyr 134. In particular the protection of Leu 36 could be also observed in the NS3/Pep4A complex (Orrù et al. 1999), suggesting a similar role of Pep4A and the P′ region of PepC in stabilizing this region. A lower susceptibility toward proteolysis was also observed for the other sites in the substrate binding region, Phe 154 and Cys 159.

However, in these experimental conditions, Tyr 6 was still recognized by chymotrypsin with nearly the same kinetics as in the isolated protein, as indicated by the rapid appearance of the two complementary fragments 2–6 and 7–186 (peaks 1 and 10 in Fig. 2B, respectively). This finding suggests that the binding of PepC strongly affects the conformation of NS3, inducing an increase in the rigidity and compactness of both protein domains. However, the presence of the peptide fragments 160–179 and 155–179 (peaks 4 and 5, respectively), indicated that in the inhibitor/NS3 complex there is a certain accessibility of the active site

The same approach was employed to investigate the conformational changes occurring in NS3 when each of the peptide inhibitors listed in Table 1 was incubated with the protease in the presence of its cofactor Pep4A. The results were then compared with the data obtained on the binary complexes described above and with those previously reported on the NS3/Pep4A heterodimer (Orrù et al. 1999). Generally the binding of inhibitors induces an overall stabilization of the cofactor/NS3 complex. This is again suggested by the increase in the enzyme/substrate NS3 ratio, needed to observe proteolysis (from 1:150 for the NS3/Pep4A to 1:100 for the inhibitor complexes). When the ternary complex NS3/Pep4A/decapeptide B was examined, the pattern of the preferential chymotryptic sites was almost identical to that observed for the NS3/Pep4A complex (Table 2) and to that observed in the analysis of the NS3/PepB binary complex described above. The only exception concerned the absence of cleavage at Leu 36, which is known to be protected by the presence of the Pep4A cofactor (Orrù et al. 1999). However, the binding of the inhibitor B to the NS3/4A complex induces partial protection of residues located in the substrate binding site, Tyr 134, Phe 154, and Cys 159 and of the region located at the extreme N-terminal portion of NS3 with a decreased kinetics of hydrolysis at Tyr 6. In Figure 3A and Table 2 for comparison, the effect for the ternary complex with the P-based-only inhibitor PepA is reported. The same protections, as for PepB, are also shown for PepA, as indicated by the decrease in the number of the fragments released in the chromatogram.

The comparison between PepA and PepB (Table2) highlights that there is not an evident role in the stabilization by

Table 2. *Preferential cleavage sites detected on NS3 and NS3/Pep4A complex either in the absence or in the presence of a 10:1 molar excess of peptide inhibitors*

Cleavage site	NS3	NS3 + PepB	NS3 + PepC	NS3/4A	NS3/4A + PepA	NS3/4A + PepB	NS3/4A + PepC
Tyr 6	++++	++++	++++	++++	++	++	+
Leu 21	+++	+++	–	+++	+++	+++	–
Leu 36	+++	+++	–	–	–	–	–
Tyr 134	++	++	–	++	–	+	–
Phe 154	++	++	+	++	–	+	–
Cys 159	++	++	+	++	–	+	–

Different cleavage sites were classified merely on qualitative kinetic evaluation.

the binding of the P′ region of PepB. Moreover, for PepA, complete protection was observed for residues Tyr 134, Phe 154, and Cys 159 at the active site, probably as a result of the strong interaction of the C-terminal carboxylate in the oxyanion hole. This important binding determinant lacks in the P-P′ decapeptide inhibitors. In both ternary complexes, the N-terminal region of NS3 is still endowed with conformational freedom, showing proteolytic accessibility at Tyr 6 and Leu 21. However, a highly compact ternary complex was obtained with the inhibitor PepC. Figure 3B shows the HPLC profiles of the aliquots withdrawn after 60 min of chymotrypsin digestion of the ternary complex NS3/Pep4A/PepC. In this case no peptide fragments formed, indicating that all the chymotrypsin sensitive sites Tyr 6, Leu 21, Leu 36, and Tyr 134, Phe 154, and Cys 159 were completely protected. In particular only a tiny amount of the peptides 3–6 and 7–186 was detected after 1-h incubation, showing that even Tyr 6 was not accessible. These results indicate that the binding of PepC caused an increase in the compactness of the inhibitor NS3/Pep4A complex with a concomitant decrease in flexibility.

To summarize our results, both the P-based and P-P′-based inhibitors, as well as the cofactor, have a specific role in the stabilization of the NS3 structure. In particular if we compare the sequences of PepA, PepB, and PepC (Table 1) and their effect on the stabilization of the corresponding complexes (Table 2), we can easily figure out that the optimized binding P′ region, Pro-Cha-Asp-Leu, has a specific role in the stabilization of the N-terminal region and in particular of the two N-terminal β-strands, which are in close contact with the cofactor Pep4A, as shown by the crystal structure in Figure 1.

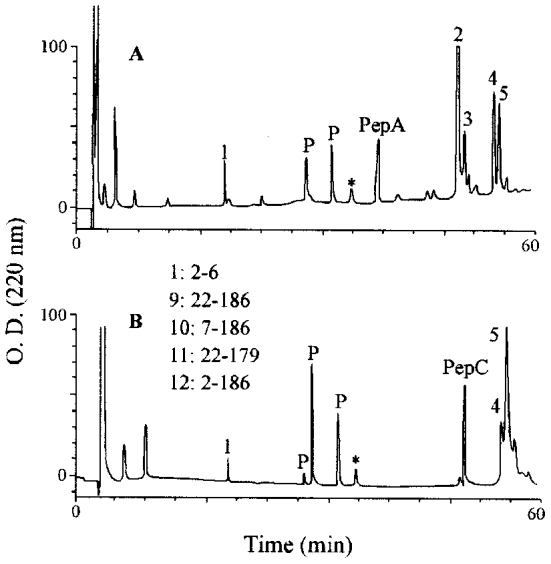

Fig. 3. Reverse-phase HPLC analysis of NS3/4A/PepA (*A*) and NS3/4A/PepC (*B*) ternary complexes incubated with chymotrypsin under controlled conditions using an enzyme/substrate ratio of 1:100 (w/w). Individual fractions were collected and analyzed by ESMS. The CHAPS peak is marked with an asterisk and those accounting for Pep4A and its proteolytic fragments are marked with P; peaks corresponding to the inhibitor PepB and PepC are also indicated.

Circular dichroism spectroscopy

The near-UV circular dichroism spectroscopy was employed as a structural probe to study the effect of binding of the inhibitors to the viral serine protease NS3. In the near-UV region between 250 and 320 nm, the CD spectrum of a protein is contributed by the aromatic side chains and it is characteristic of the protein tertiary structure. The near-UV CD spectrum of a protein is recognized as a useful technique for studying protein conformation and ligand binding.

In this study, the near-UV CD spectroscopy was used to study the binding of the decapeptide inhibitors, spanning P6-P4′, to the NS3 protease either in the presence or in the absence of 4A cofactor. The decapeptides selected in this study do not contain any aromatic residues so that any contribution in the near-UV CD region is due to the protease, namely two tryptophans and five tyrosines in the case of NS3. Any change in this region reflects an effect on the overall enzyme tertiary structure.

The results obtained on the decapeptide inhibitors were compared with those previously reported for P-spanning inhibitors. In our previous studies (Bianchi et al. 1997, 1999) we showed that cofactor complexation produces a large change in the near-UV CD spectrum of NS3 and therefore a rearrangement of tertiary structure. The P-region inhibitor binding site is contained in the C-terminal domain, whereas the P′ binding site is in the N-terminal domain. Despite the fact that the C-terminal domain is thought to be well organized structurally even in the absence of cofactor, we showed that the binding of P-based inhibitors to NS3 occurs according to an induced-fit mechanism and that various binding modes are apparent. Conversely in the presence of cofactor, all the P inhibitors bind to NS3, showing the same binding mode with a small rearrangement of NS3 tertiary structure.

Effect of decapeptide inhibitors on NS3

As already studied for the hexapeptides P-based inhibitors, we first analyzed how the P-P′ spanning decapeptide inhibitors affect the tertiary structure of the enzyme in the absence of the cofactor. Complex formation between NS3 and each inhibitor was followed in 15% glycerol, 2% CHAPS, 3 mM DTT, and 50 mM phosphate buffer, pH 7.5 at a protease concentration of 60 μM.

To study whether binding of P′ region inhibitor has any effect on the protease tertiary structure, we first compared two inhibitors that share the same P sequence but differ in the presence of the P′ part. In particular, PepA is a hexa-

peptide spanning from P6-P1; PepC is a decapeptide from P6 to P4′. Each inhibitor induces major changes in the near-UV CD spectrum of the protease, as shown in Figure 4. The resulting spectra for the two NS3/inhibitor complexes differ in the overall range, suggesting that binding in the S′ site of the enzyme can induce additional conformational rearrangements apart from those observed for the P region alone. Recent structural data by NMR have shown that the structural organization of the N-terminal domain, which contains the S′ site, is strongly dependent on the complexation to 4A. However, from these data it is evident that the decapeptide inhibitors can bind the flexible region of the N-terminal domain of the enzyme, inducing some structural organization.

In Figure 5 we compare the near-UV spectra for other decapeptide inhibitor/NS3 complexes, in particular for PepB, PepC, and PepD. Any inhibitor binding induces large differences in the overall range of the CD spectrum of the protease. Moreover these rearrangements differ among inhibitors, which in this case have different P′ sequences. So in the absence of cofactor, binding in the S′ site still endowed with a large conformational freedom is linked to the stabilization of various states of the enzyme. Again these data strongly suggest that besides the effect of the cofactor, there is also a key role played by both regions P and P′ of the substrate in the structural organization and activation of the viral enzyme.

In particular, in the case of PepB and PepC, the two peptide sequences differ only in residues at the P′ site. Here the comparison of the differences of the near-UV CD spectra of the two inhibitor/NS3 complexes reflects simply the effects of the different P′ regions of different potencies,

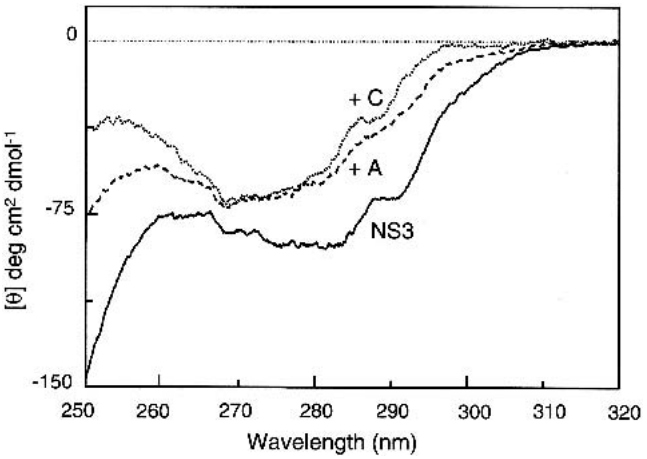

Fig. 4. Inhibitor-induced conformational change of NS3 as detected by near-UV circular dichroism in 15% glycerol, 2% CHAPS, 3 mM DTT, and 50 mM phosphate buffer at pH 7.5. The spectrum of the uncomplexed protease at 60 μM is indicated as NS3; upon formation of complexes with the hexapeptide inhibitor PepA, +A curve, and with the decapeptide inhibitor PepC, +C curve.

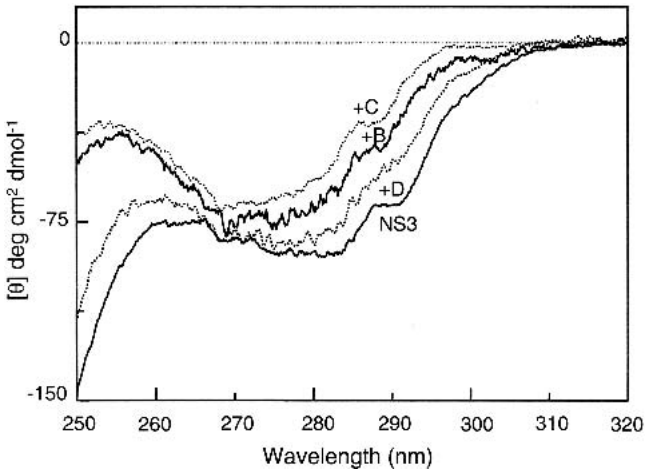

Fig. 5. Inhibitor-induced conformational change of NS3 as detected by near-UV circular dichroism. The spectrum of the uncomplexed protease at 60 μM is indicated as NS3 and upon formation of complexes with the decapeptide inhibitors: PepB, +B curve; PepC, +C curve; PepD, +D curve.

natural sequence versus combinatorially optimized, on the tertiary structure of the enzyme.

Effect of decapeptide inhibitors on the NS3/Pep4A complex

In our previous work (Bianchi et al. 1999), we showed that in the case of P inhibitors, the binding to the NS3/4A occurs according to an induced-fit model, as suggested by a modest change in the overall CD spectrum of the NS3/4A protease. We compared the effect of many P inhibitors differing in sequence and potency. Because the spectra for all the P-inhibitor/NS3/4A complexes are superimposable, binding is compatible with just one mode for the different P inhibitors. Despite so many variations, all the product inhibitors then seem to fall in the same binding class. In particular the same effect was observed for PepA, which shares the same P region of the decapeptides used in this study (Fig. 6; Bianchi et al. 1999).

The effect of the decapeptide PepC binding to the NS3J/Pep4A protease is shown in Fig. 6. The spectrum of the complex differs in the region between 250 and 290 nm from that of NS3/4A and also from the PepA/NS3/4A complex. These results suggest that the binding of the PepC in the S and S′ site occurs through an induced-fit mechanism with a rearrangement of tertiary structure of NS3/4A. The P′ region has a role in determining the structural change. In fact, binding in the S′ site produces an additional structural reorganization with respect to binding in the S site only.

In Figure 7 we can compare the near-UV CD spectra of ternary complexes for other decapeptide inhibitors from Table 1. Contrary to what was observed for S-binding inhibitors, the spectra for various decapeptide complexes are

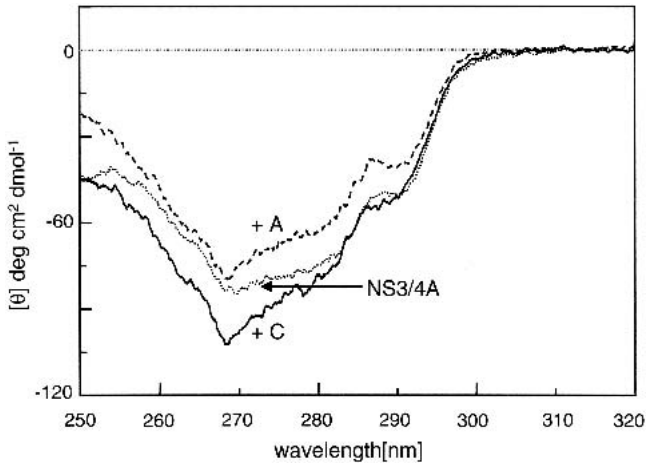

Fig. 6. Inhibitor-induced conformational change of the NS3/4A complex as detected by near-UV circular dichroism. The spectrum of the NS3/4A protease at 60 μM is indicated as NS3/4A; upon formation of complexes with the hexapeptide inhibitor PepA, +A curve, and with the decapeptide inhibitor PepC, +C curve.

quite similar but not superimposable. In particular PepC binding causes a larger increase of the intensity of the negative bands between 255 and 272 nm, whereas PepB and PepD slightly differ between 260 and 270 nm. Although these differences are quite marginal, they suggest again that slightly different rearrangements are possible for various P-binding sequences.

Therefore, whereas only one enzyme structure was attained for the ternary complex of hexapeptide product inhibitors binding in the S site, binding to the S′ site by P-P′ spanning inhibitors is linked to the stabilization of different tertiary structures of the NS3/4A enzyme. Thus different binding modes are possible when binding to the S′ site.

Discussion

In the last few years, many efforts have been directed toward the study of viral proteases as therapeutic targets for the development of antiviral agents. Viral proteases have usually a broad specificity, binding and cleaving a wide range of different peptide substrates. The mechanism by which a broad specificity can be accomplished is starting to emerge by a number of reports on different viral proteases, like HIV aspartic protease, the HCMV serine protease (LaPlante et al. 1999b), and also in the case of the NS3 serine protease of HCV (Bianchi et al. 1999; Barbato et al. 2000). A key feature of these enzymes is that they can be considered as a statistical ensemble of conformations, rapidly interconverting structures with different states populated according to their free energies.

In particular, some domains in the HIV protease, such as the flap regions that are in close contact with the substrate binding site, are known to possess extraordinarily high flexibility in the substrate-free situation. This inherent basal flexibility is a key requisite for accomodating different sequences where substrate recognition is mediated by a mechanism of stabilization of conformational states. The binding of a ligand, a substrate, or an inhibitor plays an important role in modulating the distribution of states of a protease by stabilizing certain conformations. In particular, in the case of HIV protease the binding of the inhibitor may have a significant effect on the binding site, becoming more structurally stable. Moreover, the stabilization also propagates to regions not in direct contact with the inhibitor through cooperative pathways within the protease molecule (Todd and Freire 1999).

In the case of the hepatitis C virus, the serine protease domain of NS3 folds into two six-stranded β-barrel, trypsin-like domains with the active site in a crevice between the two domains (Fig.1). The S-binding region is contained in the C-terminal β-barrel, whereas the S′ region is in the N-terminal β-barrel. Analysis in solution by circular dichroism spectroscopy and limited proteolysis-mass spectrometry have shed some insights on the mechanism of binding of both the NS4A cofactor and product inhibitors (Bianchi et al. 1997, 1999; Orrù et al. 1999). The binding of the cofactor induces a large conformational rearrangement in the tertiary structure of NS3, whereas the secondary structure is not perturbed. As a consequence of this structural change the enzyme becomes more compact and stable. Also the stabilization effect propagates toward regions remote from the cofactor binding site like the C-terminal domain. However, upon NS4A complexation, NS3 is not protected against proteolytic digestion at specific sites, essentially located at the extreme N-terminal region at residues 6, 11, 21,

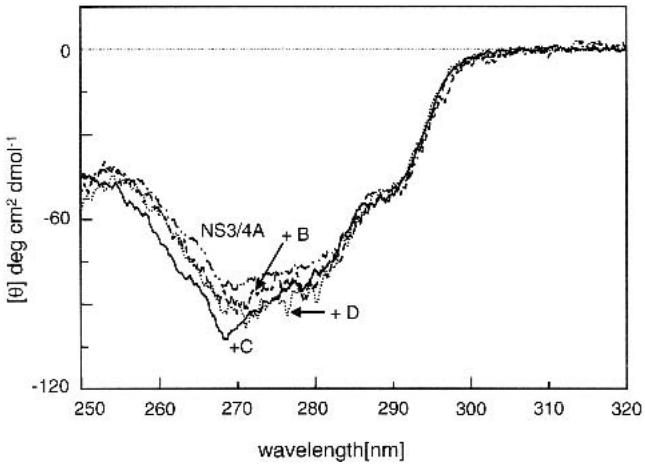

Fig. 7. Inhibitor-induced conformational change of the NS3/4A complex as detected by near-UV circular dichroism. The spectrum of the NS3/4A protease is indicated as NS3/4A, and those of the complex with the inhibitors as PepB, +B curve; PepC, +C curve; and PepD, +D curve.

24, and 26 and at the substrate binding site, residues 134, 154, and 159.

The binding of product inhibitors causes a structural change in NS3 (Bianchi et al. 1999). In the absence of cofactor, different binding modes are apparent, while in the presence of cofactor, all product-inhibitors show the same binding mode with a small rearrangement of NS3 tertiary structure. The binding of the cofactor induces an NS3/4A conformation that is already preorganized for substrate binding. However, occupancy of the S substrate binding site induces further structural rearrangements and an overall stabilization of the enzyme complex, which influences also the N-terminal region, which is directly implicated in cofactor binding. Therefore, we postulated that only through complexation of both the cofactor and the substrate, a fully competent enzyme is attained.

Our studies are in agreement with the recently published NMR studies on NS3 (Barbato et al. 2000). Despite the fact that the catalytic triad is better ordered in the presence of the NS4A cofactor, a key role in the correct alignment of the catalytic residues seems to be due to the interaction of the protease with the substrate. NMR studies on the single-chain NS3/4A show that the cofactor has a key role in the stabilization of the N-terminal domain (McCoy et al. 2001). However model-free dynamics of the complex reveals residual extensive millisecond–microsecond motion throughout the N-terminal domain. This is particularly true at the level of the same residues that are mostly affected by NS4A binding, suggesting a slow conformation exchange between the NS3–NS3/4A complex. Moreover in the complex, the N-terminal 28 residues of the NS3 protease are still endowed with high conformational flexibility.

Among studies of protease inhibitors, there are only a few examples of inhibitors that make contacts with the S′ region of the enzyme (Imperiali and Abeles 1987; Eichler and Houghton 1993; Landro et al. 1997). In the case of HCV NS3/4A, the contribution to binding of the P′ region from a natural cleavage site is small. This is because noncleavable substrate analogs extract most of their binding energy from the P side (Landro et al. 1997). An inhibitor taking advantage of S′-binding could therefore display a range of interactions with the enzyme different from the substrate and represent a novel class of NS3/4A inhibitors. Our combinatorial investigation of the P2′-P4′ residues yielded inhibitors whose potency is >600-fold with respect to inhibitors containing the natural P2′-P4′ moiety (Ingallinella et al. 2000). The recently developed P-P′ inhibitors are a useful structural probe to investigate the binding in the S′ site of the protease.

The S′ region is contained in the N-terminal domain, which is quite unstructured in the absence of 4A. The near-UV CD spectroscopy shows that in the cofactor-free NS3, binding of decapeptide inhibitors has an effect on the protease tertiary structure. The comparative analysis with inhibitors, binding only in the S site, suggests that there are additional structural rearrangements that are linked to the binding in the S′ region. Moreover, these rearrangements differ among inhibitors that have different P′ sequences. Despite the fact that the S′ is not organized in the cofactor-free protease, showing a large conformational freedom, the decapeptide inhibitors can still make contacts in this region by modulating the distribution of states of NS3 and by stabilizing different conformations. These are also reflected by the data of limited proteolysis on the decapeptide/NS3 complexes. The preferential cleavage sites in NS3 are essentially located at the extreme N-terminal region, with residues Tyr 6, Leu 21, and Leu36, and in the C domain near the substrate binding site, Tyr 134, Phe 154, and Cys 159. Upon inhibitor binding, all the complexes are generally more compact and stable and therefore it is necessary to increase the ratio of proteolytic enzyme (chymotrypsin) to substrate (NS3) to observe the same proteolytic pattern shown by NS3.

In particular for the P′-optimized PepC, complete protection is observed for Leu 21, Leu 36, and Tyr 134 sites and partial protection for Phe 154 and Cys 159. Therefore, the CD and limited-proteolysis data strongly suggest that apart from the cofactor there is a major role played by the occupancy of the substrate binding site in the structural organization and activation of the protease with effects that propagate to regions located outside the binding site.

For the ternary NS3/4A/inhibitor complexes, the comparative analysis of CD spectra for inhibitor binding in the S site and for those in which binding extends to the S′ site shows that extra binding to the S′ site causes a further rearrangement of tertiary structure of the protease. At variance with what is observed for P inhibitors, these rearrangements may slightly differ among complexes with decapeptides with diffent P′-binding sequences. This is true for different P′-natural sequences, as well as the P′-optimized sequence. Therefore, despite cofactor complexation, the N-terminal domain is still flexible, allowing different conformations. To summarize, different binding modes are possible when binding to the S′ site of the NS3/4A enzyme.

The limited proteolysis-mass spectrometry data show that binding in the S′ site can have profound effects on the stabilization of the enzyme. Again upon inhibitor binding, all the complexes are generally more stable than NS3/4A. In fact, it is necessary to increase the ratio of the chymotrypsin to substrate NS3 to observe the proteolytic cleavages. However, no major differences in the stabilization are observed between the P inhibitor PepA and the P-P′ inhibitor PepB. This is probably the result of the poor contribution to the binding energy by the P′ natural sequence of PepB. Conversely the presence of the C-terminal carboxylate in PepA, as an important binding determinant for the P inhibitor that lacks in P-P′ inhibitors could induce a strong stabilization of the active site. In particular, in our previous work, we

showed that binding in the S site, located in the C-terminal domain, also gave important contributions to the stabilization of the N-terminal domain that comprises the Pep4A cofactor binding site, as well as the S′ site. However, a residual flexibility of the N-terminal portion, in particular of the last two strands, has always been observed both in the NS3/4A complex and the P-inhibitor/NS3/4A complex. Solution structural analysis by NMR has recently confirmed the large conformational freedom endowed with this portion of the enzyme in the single-chain NS3/4A protease (McCoy et al. 2001). Conversely, in the case of the decapeptide PepC, whose sequence was investigated by combinatorial chemistry using nonnatural amino acids, the binding of P′ is associated with a structural rearrangement of the NS3 structure. A remarkable stabilization is observed not only for the active site, but also, for the first time, for the N-terminal region, in particular for Tyr 6 and Leu 21. From Figure 1 these sites are located in the same region involved in the cofactor binding, to which the S′ site also belongs, but are not in direct contact with the inhibitor.

In summary, our work shows that, as shown for other viral proteases, there are regions within the HCV NS3 protease, in particular the active site and the N-terminal domain, with less structural stability. The presence of this residual flexibility in a viral enzyme is important for mediating broad substrate specificity where substrate recognition is mediated by a mechanism of stabilization of conformational states. It was possible to develop inhibitors that target different sites on the protease surface in S and S′, also taking advantage of binding pockets present in the active site that are not exploited by the natural HCV substrates (Landro et al. 1997; Urbani et al. 1997; Zhang et al. 1997). Binding leads as a consequence to an overall stabilization of the enzyme that propagates to regions not in direct contact with the inhibitor. Differently from what has been observed in the case of binding in the S site of the NS3/4A protease, binding in the S′ site may stabilize different final states of the protease. The development of these inhibitors, which can be considered as belonging to different classes, expands the opportunities for an effective therapy against the viral proteases. A detailed understanding of the interactions between the protease and the inhibitors is required to address the issue of the most likely outcome of a clinical therapy with protease inhibitors, that is, viral resistance. An effective therapy against a viral protease would be achieved either by a combination of inhibitors of different classes or by the search among them for those that evade viral resistance (Rosin et al. 1999; Todd and Freire 1999; Dauber 2002).

Materials and methods

Enzyme preparation

The protease domain of the HCV J strain NS3 protein (amino acids 1027–1206, followed by the sequence ASKKKK) was prepared and purified as previously described (Bianchi et al. 1997; Steinkühler et al. 1998). The enzyme preparation was analyzed by mass spectrometry performed on HPLC-purified samples using a Perkin-Elmer API 100 instrument, and N-terminal sequence analysis was carried out using Edman degradation on an Applied Biosystems Model 470A gas-phase sequencer. The NS3 J preparation consisted of two components. The minor species corresponded to the intact 1–186 protease (MW 19743.1 +/– 1.5), whereas the major component showed a molecular mass of 19540.8 +/– 1.1 and was identified as a truncated form of the protease lacking the N-terminal dipeptide Met-Ala. Enzyme stocks were quantitated by amino acid analysis.

Peptide synthesis

Protected amino acids were purchased from Novabiochem, Neosystem, Bachem, or Synthetech. Peptide synthesis was performed by Fmoc/*t*-Bu chemistry (Atherton and Sheppard 1989) on Novasyn TGR (peptide amides) resin or on preloaded Fmoc-Cys(Trt)-Novasyn TGA resin. Peptide assembly was done on a Millipore 9050 Plus synthesizer, using PyBOP/HOBt/DIEA (1:1:2) activation with a fivefold molar excess of acylant over the resin groups and 1-h coupling times. The peptides were cleaved with 88% TFA, 5% phenol, 2% triisopropylsilane, and 5% water. Crude peptides were purified by reverse-phase HPLC on a Nucleosyl C-18, 250 × 21 mm, 100 A, 7 μm, using H_2O, 0.1% TFA, and acetonitrile, 0.1% TFA, as eluents. Analytical HPLC was performed on an Ultrasphere C-18, 250 × 4.6 mm, 80 Å, 5 μm (Beckman). The purified peptides were characterized by mass spectrometry, [^{1}H]-NMR, and amino acid analysis.

As protease cofactor, we used the peptide Pep4A, which encompasses the central hydrophobic domain of the HCV NS4A (amino acids 1678–1691 of the HCV polyprotein sequence) with a three-lysine tag at the N terminus to increase solubility KKKGSVVIVGRIILSGR-NH2 (Bianchi et al. 1997). The concentration of stock solutions of peptides, prepared in buffered aqueous solution, was determined by quantitative amino acid analysis performed on HCl-hydrolyzed samples.

Limited-proteolysis experiments

Limited-proteolysis experiments were performed at 25°C, in 50 mM phosphate buffer at pH 7.5, 70 mM NaCl, 2.5 mM DTT, 1% CHAPS, and 15% glycerol, using chymotrypsin as proteolytic probe. NS3/inhibitor or NS3/Pep4A/inhibitor complexes were formed by incubating either NS3 or NS3/Pep4A complex with a 10:1 molar excess of the individual inhibitor at 25°C for 15 min prior to proteolytic enzyme addition. Each inhibitor complex was digested using a 1:100 (w/w) enzyme to substrate ratio; NS3 or NS3/4A complex were digested using a 1:150 (w/w) ratio.

The extent of the enzymatic hydrolysis was monitored on a time-course basis by sampling the incubation mixtures at appropriate time intervals, followed by HPLC fractionation of proteolytic fragments on a Vydac C18 column. Peptides were eluted by means of a linear gradient from 5% to 65% of acetonitrile in 0.1% TFA over 73 min. Identification of individual fragment samples was performed by injecting the HPLC fractions into an electrospray ion source (kept at 80°C) at a flow rate of 5 μL/min, on a Bio-Q triple quadrupole mass spectrometer (Micromass). Data were analyzed using the MASSLYNX program. Mass calibration was performed by means of the multiply charged ions from a separate injection of horse heart myoglobin (average molecular mass 16,951.5 Da); all masses are reported as average mass.

Circular dichroism spectroscopy

Circular dichroism measurements were performed using a Jasco J-710 spectropolarimeter equipped with a cell holder thermostatically controlled by a circulating water bath. Measurements were recorded at 15°C, with an 8-sec time constant and 5 nm/min scan speed and spectra were averaged for two acquisitions. The protein concentration was 1.2 mg/mL, and spectra were collected with rectangular quartz cells of 1-cm path length in the near-UV region (320–250 nm) and 0.01-cm path length in the far-UV region (250–190 nm). The concentration of both protein and peptide stock solutions was determined by quantitative amino acid analysis. Results are reported as mean residue ellipticity [θ] having units of deg cm^2 $dmol^{-1}$ and were calculated using a mean residue weight of 106 Da for NS3. The complexes were formed by incubating the protease with increasing amounts of the peptide inhibitors either in the presence or in the absence of Pep4A for 10 min at 15°C. The complexes were studied in 50 mM sodium phosphate buffer at pH 7.5, 15% glycerol, 2% CHAPS, and 3 mM DTT. Spectra were recorded before and after the addition of Pep4A and/or inhibitors and routinely corrected for the background signal and for dilution effects. By following the ellipticity as a function of peptide concentration it was possible to derive a stoichiometry of 1:1 for each inhibitor with respect to the protease.

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