The dynamic properties of the Hepatitis C Virus E2 envelope protein unraveled by molecular dynamics

Daniela Barone\textsuperscript{a,b,†}, Nicole Balasco\textsuperscript{a,b,†}, Ida Autiero\textsuperscript{a} and Luigi Vitagliano\textsuperscript{a**}

\textsuperscript{a}Institute of Biostructures and Bioimaging, C.N.R., Naples I-80134, Italy; \textsuperscript{b}Dipartimento di Scienze e Tecnologie Ambientali Biologiche e Farmaceutiche, Seconda Università di Napoli, Caserta 81100, Italy

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Hepatitis C Virus (HCV) is one of the most persistent human viruses. Although effective therapeutic approaches have been recently discovered, their use is limited by the elevated costs. Therefore, the development of alternative/complementary strategies is an urgent need. The E2 glycoprotein, the most immunogenic HCV protein, and its variants represent natural candidates to achieve this goal. Here we report an extensive molecular dynamics (MD) analysis of the intrinsic properties of E2. Our data provide interesting clues on the global and local intrinsic dynamic features of the protein. Present MD data clearly indicate that E2 combines a flexible structure with a network of covalent bonds. Moreover, the analysis of the two most important antigenic regions of the protein provides some interesting insights into their intrinsic structural and dynamic properties. Our data indicate that a fluctuating β-hairpin represents a populated state by the region E2\textsuperscript{412−423}. Interestingly, the analysis of the epitope E2\textsuperscript{427−446} conformation, that undergoes a remarkable rearrangement in the simulation, has significant similarities with the structure that the E2\textsuperscript{430−442} fragment adopts in complex with a neutralizing antibody. Present data also suggest that the strict conservation of Gly436 in E2 protein of different HCV genotypes is likely dictated by structural restraints. Moreover, the analysis of the E2\textsuperscript{412−423} flexibility provides insights into the mechanisms that some antibodies adopt to anchor Trp437 that is fully buried in E2. Finally, the present investigation suggests that MD simulations should systematically complement crystallographic studies on flexible proteins that are studied in combination with antibodies.

Keywords: molecular dynamics; protein flexibility; conformational change; viral proteins; antibody binding

Introduction

Hepatitis C virus (HCV) is one of the most persistent human viruses (Falkowska, Kajumo, Garcia, Reinus, & Dragic, 2007; Helle et al., 2007, 2010; Pantua et al., 2013). It infects 185 million of people worldwide and is frequently able to escape the immune system of HCV-infected patients. Current estimates indicate that as many as 80% of these patients develop severe liver diseases like cirrhosis and hepatocellular carcinoma (Bowen & Walker, 2005). Although effective therapeutic approaches, based on specific inhibitors of HCV proteins NS3/4A and NS5B, have been recently discovered, their use is limited by the elevated costs of these drugs (Kwong, Mcnair, Jacobson, & George, 2008; Matthews & Lancaster, 2012). In this scenario, an urgent medical need is the development of alternative/complementary strategies such as those based on the use of vaccines. In this framework, it is obvious that a better understanding of the molecular and structural mechanisms underlying virus–host interaction will certainly facilitate the design of effective vaccines.

HCV is a positive-stranded RNA virus belonging to the Flaviviridae family. Its genome encodes a single polypeptide of ~3000 amino acid residues. This precursor form is processed by host and viral proteases into 10 polypeptides which include two envelope proteins (E1 and E2). The envelope proteins are assembled in a non-covalent heterodimer through their transmembrane domains (Cocquerel, Wychowski, Minner, Penin, & Dubuisson, 2000; Deleersnyder et al., 1997; Dubuisson, 2000). The envelope complex thus formed is essential for mediating the HCV entry into hepatocytes through the binding with receptors on the host cell surface. Indeed, it has been reported that E2 is able to bind with high affinity to the extracellular loop of CD81 receptor that is expressed in several cell types including hepatocytes (Pileri et al., 1998). Since E2 plays a key role in the virus entry, it represents the most immunogenic region of the HCV and one of the main target of neutralizing antibodies (nAb) (Keck et al., 2012). This feature makes the E2 glycoprotein an essential starting point for the design of new vaccines. Although several regions of the protein are the
target of neutralizing mAbs, two major antigenic regions have been identified in the E2 sequence (Allander et al., 2000; Ball, Tarr, & McKeating, 2014; Drummer, Boo, Maerz, & Poumbourios, 2006; Drummer, Wilson, & Poumbourios, 2002; Duan et al., 2012; Flint et al., 1999; Hsu et al., 2003; Johansson et al., 2007; Law et al., 2008; Mancini et al., 2009; Owsianka et al., 2005; Owsianka et al., 2006; Perotti et al., 2008; Roccascecca et al., 2003; Triyatni et al., 2002; Zhang et al., 2007). These regions have been identified in the recognition of the human CD81 receptor such as Thr416, Trp420, Gln431, Asn434, Trp437, Leu438, Trp529, Gly530, and Asp535 (Owsianka et al., 2006; Perotti et al., 2008). Although HCV E2 has been the subject of extensive characterizations for decades, structural data on this protein have become available only recently. Indeed, in the last few years, a number of impressive crystallographic analyses have shed light on the E2 global structural features as well as on the interaction of E2 fragments with neutralizing/non-neutralizing mAbs (Deng et al., 2014; Deng et al., 2013; Khan et al., 2014; Kong et al., 2013; Kong, Giang, Nieusma et al., 2012; Kong, Giang, Robbins et al., 2012; Krey et al., 2013; Li et al., 2015; Meola et al., 2015; Pantua et al., 2013; Potter et al., 2012). Global features of the E2 protein have been derived from the crystallographic studies of its complexes with antibodies. Although different constructs have been used in these two independent studies they provide convergent information (Khan et al., 2014; Kong et al., 2013). The core of the protein is constituted by a central β sandwich surrounded by loops, short helices, and β sheets. Interestingly, E2 is characterized by a rather limited content of secondary structure (38%) (Kong et al., 2013). The overall fold of the protein is likely stabilized by an intricate network of disulfide bridges. Only the structure corresponding to the larger construct (PDB ID: 4MWF) provides information on the epitope E2427−446, also defined as antigenic site AS434 by Kong, Jackson, Wilson, and Law (2015), whose central part adopts a helical conformation (residues 436−442). In both of the crystallographic structures no structural information is provided for the epitope E2412−423, also defined as antigenic site AS412 by Kong et al. (2015), being either absent in the protein construct (PDB ID: 4WEB) or disordered in the crystal state (PDB ID: 4MWF) (Khan et al., 2014; Kong et al., 2013).

Interesting insights into the recognition mechanism of E2 by mAbs have been provided by crystallographic analyses of complexes between mAbs and peptides corresponding to the epitopes E2412−423 and E2427−446. Initial crystallographic studies indicated that mAbs such as AP33, HCV1, MRCT10.v362, and hu5B3.v3 bind the epitope E2412−423 assuming a β-hairpin conformation (Kong, Giang, Nieusma et al., 2012; Kong, Giang, Robbins et al., 2012; Pantua et al., 2013; Potter et al., 2012). We have observed a β-hairpin conformation in the crystal structure of a cyclic variant of epitope E2412−423 bound to a non-neutralizing mAb (Sandomenico et al., 2016). Intriguingly, very recent structural studies indicate that other mAbs such as HC33.1 and 3/11 anchor the epitope E2412−423 assuming a rather extended conformation (Li et al., 2015; Meola et al., 2015).

Crystal structures of the mAbs bound to fragments of the epitope E2427−446 also provided interesting information (Deng et al., 2014; Deng et al., 2013; Krey et al., 2013). Of particular interest is the comparison of the interaction of fragments of the epitope E2427−446 region with neutralizing (mAb#8) and non-neutralizing (mAb#12) mAbs which highlighted the possibility that the local E2 region may adopt an open state which is alternative to the closed state observed in the crystal structure (Deng et al., 2014). In this scenario, here we report an extensive analysis of the intrinsic dynamic and structural properties of the E2 protein by performing a molecular dynamics (MD) simulation. In particular, starting from the structure of the protein in complex with the mAb AR3C (Figure 1), we evaluated the evolution of the protein structure upon mAb removal. Our data provide interesting clues on the global and local intrinsic dynamic features of the protein that could be exploited in future studies aimed at developing HCV vaccines.

**Materials and methods**

**Notations and molecular modelling**

The HCV envelope E2 protein includes residues 384−746 of the viral polypeptide. As mentioned above, two independent crystallographic structures of a truncated variant of E2 in complex with antibodies have been

![Figure 1. Cartoon representation of the structure of the Hepatitis C Virus Envelope Glycoprotein E2 (blue) bound to the broadly neutralizing antibody AR3C (red) refined at 2.64 Å resolution (PDB ID: 4MWF). The E2 antigenic region 427−446 is colored in cyan. The disulphide bridges are rendered in stick representation.](Image 318x143 to 548x270)
recently solved. The structure of the E2 longest variant (residues 412–645) extracted from the complex that the E2 core forms with the broadly neutralizing antibody AR3C (PDB ID: 4MWF) (Kong et al., 2013) (Figure 1), was used as starting model in the MD simulation. In this variant the protein sequence corresponds to the HCV E2 genotype 1a (H77 strain). This E2 core construct lacks the region spanning residues 460–485, whereas several fragments (residues 412–420, 453–459, 486–491, 574–577, and 586–596) are disordered in the crystallographic model. The core of the protein contains 16 cysteine residues which are involved in the formation of 8 disulfide bonds (Cys429–Cys503, Cys452–Cys620, Cys459–Cys486, Cys494–C564, Cys508–Cys552, Cys569–Cys581, Cys585–Cys597, and Cys607–Cys644). The disulfide Cys459–Cys486 is disordered in the experimental structure.

The disordered regions in the crystallographic E2 model were generated using information derived either from complexes of E2412–423 fragments and mAbs or standard molecular modeling techniques. Several independent crystallographic structures of mAb-bound E2412–423 fragments indicate that they adopt a rather conserved β-hairpin structure (Kong, Giang, Nieusma et al., 2012; Kong, Giang, Robbins et al., 2012; Pantua et al., 2013; Potter et al., 2012). Although more recent investigations have shown that these fragments are also able to adopt more extended conformation when bound to other mAbs (Li et al., 2015; Meola et al., 2015), the potential tendency of E2412–423 to form β-structure is confirmed, at least for the region encompassing residues 412–415, by secondary structure prediction analyses performed using the PSIPRED server (http://bioinf.cs.ucl.ac.uk/psipred/) (Supplementary Figure S1). Similar indications have been provided by the PEP-FOLD server (http://bioserv.rpbs.univ-paris-diderot.fr/services/PEP-FOLD/) (data not shown). Therefore, the E2 region 412–423 was modeled as a β-hairpin that makes no contact with the rest of the protein.

The regions including residues 453–459 and 486–491 were modeled as coil to generate the disulfide bond between Cys459 and Cys486. The missing regions corresponding to residues 574–577 and 586–596 were not rebuilt since the two disulfide bridges Cys569–Cys581 and Cys585–Cys597 assure the covalent connectivity in the E2 core structure. Therefore, the final generated model consists of four polypeptide chains linked by disulfides bridges: residues (i) 412–459, (ii) 486–573, (iii) 578–585, and (iv) 597–645. The E2 protein shows a number of glycosylation sites. Those occurring in the present model are Asn417, Asn423, Asn430, Asn448, Asn532, Asn540, Asn556, Asn623, and Asn645. However, the N-linked glycans were not considered in this simplified model. The potential effect of glycosylation has been considered in interpreting the MD results (Goffard et al., 2005).

Simulation procedures
GROMACS software package 4.5.5 (Van Der Spoel et al., 2005) was used to perform a MD simulation under AMBER99SB force field. The starting model of the E2 core was immersed in a triclinic box (6.265 × 7.425 × 6.669 nm³) filled with 9257 TIP4P water molecules. The simulation was run applying periodic boundary conditions. Equilibration of the system was conducted in order to stabilize the temperature (300 K) and the pressure (1 atm). Energies were initially minimized by fixing the protein atoms and then without restraints. The Particle Mesh Ewald method (grid spacing of 0.12 nm) was used to calculate the electrostatic interactions, whereas a cut-off of 10 Å was applied to treat Lennard–Jones interactions. The LINCS algorithm was used to constrain bond lengths. The timescale of the simulation was 500 ns with a time step of 0.002 ps.

The collective motions of the protein in the MD simulation were examined by performing an essential dynamics (ED) analysis. The covariance matrix of the coordinate fluctuations was diagonalized to obtain eigenvectors and eigenvalues. The convergence of the simulation in the essential space has been checked by calculating the root mean square inner product (RMSIP) between the first 10 eigenvectors of the two halves of the equilibrated trajectory (Amadei, Ceruso, & Di Nola, 1999; Amadei, Linssen, & Berendsen, 1993; Merlino, Vitagliano, Ceruso, & Mazzarella, 2002; Merlino, Vitagliano, Ceruso, & Mazzarella, 2003).

Trajectories were checked to assess the quality of the simulation using GROMACS routines and the program VMD (Humphrey, Dalke, & Schulten, 1996). H-bond interactions have been identified based on cut-offs for the angle hydrogen-donor–acceptor (~30°) and the distance donor–acceptor (~3.5 Å) using GROMACS utilities (Van Der Spoel et al., 2005). The GROMACS tool g_rmsf was used in order to calculate the root mean square fluctuations (RMSF) of the Ca atoms and the root mean square deviations (RMSD) per residue. In particular, the RMSD per residue is the deviation (computed on the Ca atoms) of residues of the average structure, computed in the equilibrated region of the trajectory, relative to the crystallographic model.

Results
Overall structural stability and flexibility of E2 core
The stability of the model throughout the simulation was evaluated by analyzing the time evolution of several structural indicators. As shown by the RMSD, computed on the Ca atoms of the secondary structure elements of the trajectory frames relative to the starting model (Figure 2(A)), the system reaches a rather stable state after ~200 ns. The secondary structure elements are well
preserved along the simulation (Figure 2(B)). The analysis of other indicators as the radius of gyration (~16.8 Å) and the number of hydrogen bonds (~100) which were both rather constant along the simulated time confirmed the overall stability of the system (Supplementary Figure S2). An ED analysis was performed to evaluate the convergence of the simulation and to gain insights into the collective motions of the protein (see also below). The convergence was checked dissecting the equilibrated region of the trajectory in two halves: 200–350 ns and 350–500 ns. Then, independent ED analyses were performed. The similarity of the motions in these two regions was evaluated by comparing the resulting eigenvectors (see Methods for details). The rather high value of the RMSIP (0.68) computed using the first 10 eigenvectors obtained in these two halves of the equilibrated trajectory indicates that the protein displays similar overall motions, thus suggesting that a good convergence was achieved in the simulation.

In order to shed light on the intrinsic dynamic properties of the E2 protein devoid of mAb partners, we calculated RMSD values against the starting model extracted from the PDB entry 4MWF (Kong et al., 2013) and RMSF values in the equilibrated region of the trajectory (see Methods for details).

The RMSD values calculated in the equilibrated region of the trajectory (approximately 4.0 Å for the residues belonging to secondary structure elements) suggest that the model undergoes significant structural transitions in the MD simulation (Figure 2(A)). The inspection of the RMSD per residue values, which show the deviations from the crystallographic model of different protein regions, indicates that the highest displacements occur for the regions 432–451 and 520–530 (Figure 3(A)). It is worth mentioning that these areas encompass the epitope E2427–446 and the region 523–540, which is also a target of neutralizing mAbs (Ball et al., 2014; Owsianka et al., 2008). This observation is not surprising considering that (a) the region 427–446 contains several residues involved in the interactions with the AR3C mAb and (b) the region 523–540 is proximal to the mAb recognition site. Interestingly, this result indicates that the intrinsic conformations of these regions in the isolated E2 protein are different from those detected in the crystal structure of the complex. The implications of this finding are analyzed in a specific section below.

In order to gain insights into protein flexibility, we calculated the RMSF values on the Cα atoms in the equilibrated region of the trajectory. This analysis reveals that...
the simulated model is endowed with an intrinsic considerable flexibility. As expected, residues belonging to the secondary structure elements of the protein display lower RMSF values (0.5–1.0 Å), whereas high values (up to 6.5 Å) are observed for residues belonging to loops (Figure 3(B)). Although epitopes E2412–423 and E2427–446 are endowed with a significant secondary structure content, their flexibility is higher than that observed for other structured elements. Indeed the RMSF values for these two regions range from 1.0 to 2.0 Å. Even larger fluctuations are in correspondence of the region 523–540. The flexibility of the latter region is somewhat expected as it does not contain secondary structure elements.

These results have been corroborated by an ED analysis which was conducted using the structures of the equilibrated region of the trajectory (200–500 ns). The inspection of eigenvalues suggests that the overall motions of the protein may be satisfactorily described by a limited number of eigenvectors. Indeed, the first 10 eigenvectors represent the 80% of the total fluctuations. Moreover, the first and the second eigenvector cover the 31% and the 14% of the total motions. The visualization of the protein motion along the first and the second eigenvectors in a film-like fashion (Figure 4) confirms that the region 523–540 is the most flexible one. It is followed by epitopes E2412–423 and E2427–446.

In conclusion, these results suggest that, although the protein essentially retains its folded state along the MD simulation, the E2 core is endowed with an elevated level of global and local flexibility. A detailed analysis of the two major E2 antigenic regions (E2412–423 and E2427–446) is reported below.

**Dynamics of the epitope E2412–423**

The epitope E2412–423 represents the most highly conserved antigenic site of the E2 protein. Over the years, several mAbs targeting this region have been developed (Kong, Giang, Nieuima et al., 2012; Kong, Giang, Robbins et al., 2012; Li et al., 2015; Meola et al., 2015; Pantua et al., 2013; Potter et al., 2012). Due to the absence of structural data on E2412–423 in the protein context, in our model this region has been modeled as a β-hairpin on the basis of predictive analyses (Supplementary Figure S1) and of data derived from complexes that E2412–423 fragments form with mAbs (see Methods for details).

MD data revealed that the β-structure is well preserved along the simulation although some H-bond interactions of the β-hairpin sporadically lost (Figure 2(B)). In addition to the main chain H-bonds, crystallographic analysis has suggested that the stability of this motif also relies on a H-bond formed by the Asn415 side chain (Oδ1 atom) and Gly418 nitrogen atom. Notably, this interaction is well preserved in the trajectory structures (Supplementary Figure S3). However, although structurally stable, the E2412–423 hairpin motif is endowed with a significant level of flexibility in the MD simulation, as indicated by both its high RMSF values (Figure 3(B)) and its wide fluctuations observed in the ED analysis (Figure 4). In principle, the high mobility of this epitope region may be ascribed to the deletion of the N-terminal portion (residues 384–411). However, taking into account that this fragment corresponds to the HVR1 (highly variable region 1) region, it is likely that it has a minor impact on the epitope conformation.
A deeper analysis of the mobility of the entire E2\textsuperscript{412–423} hairpin indicates that starting from a fully exposed state in the starting model (see Methods) it tends to get closer to the E2\textsuperscript{427–446} epitope. As shown in Figure 5, the distance between the centers of mass of the two epitopes significantly decreases in the simulation. Although this observation may be partially biased by the lack of glycosylation on Asn\textsuperscript{417} and Asn\textsuperscript{423}, the dynamic interactions between these two epitopes may be related to the interference sometimes observed between pairs of mAbs each targeting one of these two regions.

It is worth mentioning that E2\textsuperscript{412–423} residues typically recognized by mAbs targeting the epitope remain fully exposed in the simulation structures. In particular, it has been shown that mAbs recognizing the E2\textsuperscript{412–423} hairpin specifically bind the peptide face constituted by the side chains of residues Leu\textsuperscript{413}, Asn\textsuperscript{415}, and Trp\textsuperscript{420}. The analysis of the solvent accessible surface (SAS) of these side chains (Supplementary Figure S4) indicates that they are exposed to the solvent in the simulation, and therefore accessible to these mAbs.

**Dynamics of the epitope E2\textsuperscript{427–446}**

The E2 region 427–446 is recognized by several, either neutralizing or non-neutralizing, mAbs (Deng et al., 2014; Deng et al., 2013; Krey et al., 2013). One of the neutralizing mAb that targets this region is AR3C (Figure 1). Therefore, as anticipated above, it is not surprising that E2\textsuperscript{427–446} is the protein region which presents the highest variation in the MD simulation when compared to the starting crystallographic model (Figure 3(A)). This portion also displays a significant flexibility (Figure 3(B)). It is worth mentioning that Asn\textsuperscript{430}, which is glycosylated in the wild-type protein, is highly exposed to the solvent throughout the simulation (data not shown). This is in line with the marginal influence that the glycosylation of this residue has on the protein structure and the virus infectivity (Goffard et al., 2005).

One important residue located in this region is the strictly conserved Gly residue in position 436. Site-directed mutagenesis of this residue to serine, proline or alanine revealed a significant decrease in the virus ability to penetrate the host cells (Drummer et al., 2006). It has been recently proposed that this residue is important for endowing this region with the flexibility required for functional/structural transitions. The analysis of trajectory structures suggests a complementary but distinct explanation for the structural role played by this residue. Indeed,
the inspection of Gly436 conformations in trajectory frames clearly indicates that this residue populates Ramachandran regions that are unusual or even forbidden to all other protein residues (Figure 6). Indeed, starting from \((\phi, \psi)\) values of \((-60^\circ, 30^\circ)\) in the crystallographic model, this residue specifically assumes conformations with positive values of \(\phi\) angle in all simulation structures. In this framework, Gly436 appears to play an important structural role. Any replacement of this residue likely perturbs the structural integrity of the protein thus impairing its functional role.

A comparative analysis of the complexes that mAbs form with the E2 core and with peptide fragments of the region 427–446 highlights a remarkable variability in the recognition modes. In particular, the residues of the E2 core interacting with AR3C are Leu427, Cys429, and Glu431 (Kong et al., 2013). On the other hand, HC84-1 and HC84-27 mAbs recognize a region which is shifted at the C-terminus of the epitope. In particular, these mAbs specifically bind the fragment Leu441-Gln444 and Lys446. Finally, the neutralizing mAb#8 and the non-neutralizing mAb#12 bind an intermediate region of the epitope which includes Glu431, Asn434, Trp437, and Lys446. The binding mode of these latter mAbs is particularly puzzling since (a) mAbs targeting these residues display radically different neutralizing abilities and (b) key residues of the recognition (Trp437 and Leu438) are fully buried by the loop 8 residues (556–560) in the crystal structure of the E2 core. The former observation led Deng et al. to suggest that the neutralizing/non-neutralizing effects were related to the different juxtaposition of the different portions of the epitope (helix and the loop located at its N-terminal end) in the complexes of the peptides with mAb#8 and mAb#12 (Deng et al., 2014; Deng et al., 2013). On the basis of the latter observation the same authors hypothesized that the local structure of the protein may present an alternative state that exposes Trp437 and Leu438 residues to the solvent.

In order to gain insights into these peculiar aspects we carefully analyzed the plasticity of this region in the trajectory structures. We compared the average MD structure calculated in the equilibrated region of the trajectory of the mAb-free E2 core with the structures of this region observed in the complexes formed with mAb#8 and mAb#12. As shown in Supplementary Table S1 and Figure 7(A), the RMSD values calculated on the C\(^\alpha\) atoms of the fragment 434–442 clearly indicate that the MD structure is more similar to that observed in the complex with the neutralizing mAb#8 (RMSD value of \(-2\) Å) than that adopted in the complex with the non-neutralizing mAb#12 (RMSD value of \(-3\) Å). This finding suggests that the neutralizing antibody binds E2\(^{427\text{-}446}\) in a conformation that is likely reminiscent of the intrinsic structural state adopted by this region in the mAb-free state. The latter observation is also evident from the superimposition of the different structures of this fragment (Figure 7(B)). This conclusion is corroborated by the analysis of Gly436 conformations explored along the simulation with those adopted in the crystallographic structures of the E2\(^{427\text{-}446}\) peptides (Figure 6). The analysis unveiled that Gly436 of the simulated system gradually assumes \((\phi, \psi)\) values closer to those adopted in the peptide bound to the neutralizing mAb#8 (\(75^\circ, -177^\circ\)) rather than in the peptide bound to the non-neutralizing mAb#12 (\(98^\circ, 136^\circ\)).

Subsequently, we calculated in trajectory structures the SAS of the residue Trp437 that is buried in the crystallographic structure of the E2 core but nevertheless anchored by interacting residues (Figure 8(A)). On average we observe a significant increase in the SAS for this residue along the trajectory. Indeed, the accessible surface of the Trp437 significantly increases from the starting value (10%) to values that on average are in the range of 30–40%. Although sporadically, the Trp437 SAS may reach values close to 75%. The opening of the local structure is confirmed by the analysis of other parameters such the distance between the C\(^\alpha\) atoms of Trp437 and the central residue of the loop 8 (Thr558). As shown in Supplementary Figure S5 this distance increases from approximately 9 Å up to 15 Å, thus leading to the exposure of this residue that can potentially interact with the antibody. The opening of the site is shown in Figure 9 where the trajectory structure exhibiting the highest exposure of Trp437 is reported. On the
other hand, Trp616, which is spatially close to Trp437 and has been reported to be recognized by the mAb 2C21 (Deng et al., 2015), is fully buried in the trajectory structures (Figure 8(B)).

**Discussion**

The recent characterization of the E2 structure represents a fundamental step for understanding the mechanism of HCV invasion and for the structure-based design of innovative therapeutic agents. This structure has highlighted a number of unexpected features including the absence of any similarity with class II fusion proteins. The E2 core structure is characterized by a rather low secondary structure content (38%) (Khan et al., 2014; Kielian, 2006; Kong et al., 2013). Although these crystallographic studies combined with the structural analyses of E2 epitopes bound to mAbs (Deng et al., 2014; Deng et al., 2013; Kong, Giang, Nieuwma et al., 2012; Kong, Giang, Robbins et al., 2012; Krey et al., 2013; Li et al., 2015; Meola et al., 2015; Pantua et al., 2013; Potter et al., 2012) have enormously expanded our current knowledge of HCV surface proteins, there are some issues that need to be addressed. In this framework, the main aim of the present study was to gain further insights into the global and local intrinsic structural/dynamic properties of HCV.

Figure 7. Time evolution of the RMSD values calculated on the reduced portion E2$^{434-442}$ of trajectory frames against the starting structure (in red), and the two crystallographic structures bound to either neutralizing (in blue) or non-neutralizing (in green) antibodies are reported in panel A. A front view of the superimposition of the E2$^{434-442}$ fragment in the crystallographic structure (red), in the complexes bound to the neutralizing mAb#8 (blue) and the non-neutralizing mAb#12 (green) antibodies, and in the average MD structure (grey) is shown in panel B. The location of Gly-436 residues for all structures has been indicated with spheres.

Figure 8. Percentage of the side-chain solvent-accessible surface (SAS) of Trp437 (A) and Trp616 (B) residues as a function of time.
E2. The present MD analysis conducted on the protein freed of the AR3C mAb clearly indicates that the E2, although highly flexible, is stable in the simulation timescale. Indeed, the limited level of secondary structure is compensated by the presence of eight disulfide bridges. The crucial role of the disulfide bridges in endowing the protein with a well-defined fold is demonstrated by the extremely low intrinsic propensity of the E2 sequence to adopt secondary structure elements (Supplementary Figure S1). This combination of a flexible fold with a network of covalent bonds may represent an important feature of the protein for the interaction with its biological targets. In fact, as frequently observed for promiscuous proteins, the flexibility is an important element as it allows the ad hoc exposure of sticky regions.

The analysis of the two most important antigenic regions of the protein provides some interesting insights into their intrinsic structural and dynamic properties. The region E2412−423 behaves in the simulation as a fluctuating β-hairpin. Although the simulation timescale is probably too limited to provide final answers on the conformational preferences of this E2 region, present data suggest that the hairpin motif here considered and observed in some complexes of peptides with mAbs is (at least) a metastable state for the E2412−423. It is worth mentioning that this observation is not in contrast with the crystallographic analysis that indicates that this region is disordered in the crystal state as this disorder may be generated by the overall high mobility of E2412−423. The mobility of E2412−423, which occasionally becomes proximal to the other major antigenic site E2427−446 may be responsible for the interference of mAbs targeting different epitopes of E2.

The region corresponding to the epitope E2427−446 is the one that undergoes the most significant structural rearrangements in the simulation. Interestingly, recent investigations have highlighted that the structure/dynamics of this region is crucial for the recognition of mAbs and for determining their either neutralizing or non-neutralizing activity. The present analysis provides some interesting clues on these issues. Our data clearly indicate that, in the protein freed of the mAbs, this region intrinsically tends to adopt a conformation that is closer to that observed for the corresponding fragment in the complex with a neutralizing mAb (mAb#8) than with the non-neutralizing one (mAb#12). Moreover, present data suggest that the strict conservation of Gly436 may be dictated by structural restraints. Since this residue essentially assumes conformations that are unusual or even forbidden to other residues its substitution would undermine the structural integrity of the E2 protein.

One of the puzzling issue related to E2427−446 recognition is the observation that some mAbs anchor the side chain of Trp437 that is fully buried in the crystal structure of E2. On the basis of this finding the possibility that an alternative more opened structure of this region could exist has been raised. Our findings suggest that this region is endowed with a significant flexibility. Although we did not observe major rearrangements, the analysis of the trajectory structures indicates that the Trp437 side chain is occasionally exposed to the solvent and therefore accessible to mAbs binding. Interestingly, the side chain of the nearby Trp616 is never exposed to the solvent in the simulation, although this residue has been considered the target of mAbs such as 2C21 and HC84-27 (Deng et al., 2015; Keck et al., 2012; Krey et al., 2013). The involvement of Trp616 in the binding of this mAb has been deduced from an Ala-scan mutagenesis analysis which showed that the mutated E2 variants no longer recognized 2C21. Present observation suggests an alternative possibility. Indeed, the location of Trp616 side chain in the hydrophobic core of E2 and its limited

Figure 9. Cartoon representation of the crystallographic structure of the E2 protein core (PDB ID: 4MWF) (A), and of the structure corresponding to trajectory frame t = 240.354 ns (B). The Trp437 and Trp616 residues are shown as sticks.
mobility indicates that it may be essential for the protein integrity. Therefore, its replacement with Ala may undermine the E2 structure and thus its ability to bind these antibodies. This is in line with the protein destabilization observed upon replacement of residues in the region 611–631 (Rychlowska et al., 2011).

Finally, the present investigation clearly shows the potential of MD in complementing crystallographic data. Since proteins that are difficult to be crystallized are frequently studied in combination with mAbs, MD analyses on these proteins freed of the antibody will likely provide insightful information on the intrinsic properties of these systems and, therefore, should systematically follow the crystallographic studies.

Abbreviations

HCV Hepatitis C Virus
E2 envelope glycoprotein 2
NS non-structural proteins
CD81 cluster of differentiation 81
PDB protein data bank
nAb neutralizing antibodies
mAb monoclonal antibodies
MD molecular dynamics
RMSF root mean square fluctuation
RMSD root mean square deviation
RMSIP root mean square inner product
E2412–423 E2 residues 412–423
E2427–446 E2 residues 427–446
HVR1 highly variable region 1

Supplemental material

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ORCID

Nicole Balasco http://orcid.org/0000-0001-7862-9448

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