

Determination of Pore-Lining Residues in the Hepatitis C Virus p7 Protein

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ABSTRACT The p7 protein from hepatitis C virus is critical for the assembly and secretion of infectious virus, making it an attractive drug target. It is thought to be a viroporin with a demonstrated ion channel activity when reconstituted into planar lipid bilayers. Electron microscopy experiments suggest that p7 oligomers coexist as hexamers and heptamers. Proposed models of p7 oligomers assume the N-terminal helix to be the pore lining helix. Here, we demonstrate, via electrophysiology, that Cu^{2+} has an inhibitory effect on the p7 ion channel and that the amino acid responsible for this inhibition is one histidine in each monomer. This information coupled with the p7 sequence data suggests that the N-terminal helix of p7 does indeed form the transmembrane pore and that this histidine is pore-lining. The information will aid in the construction of oligomeric pore-models and the interpretation of electron microscopy data.

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Hepatitis C virus (HCV) infects more than 170 million people worldwide and is one of the major causes of liver disease including chronic hepatitis, cirrhosis, and hepatocellular carcinoma (1). No vaccine is available and the current treatment, pegylated interferon- α and ribavirin is expensive and ineffective in ~50% of patients (2). Therefore, there is great desire to identify alternative drug targets. The virally encoded p7 protein, shown to be crucial for the secretion of infectious particles, is one such target (3,4).

The HCV p7 monomer is a hydrophobic protein of 63 amino acids (5) located at the junction of the structural and nonstructural (replicative) proteins of the HCV polyprotein. Two topologies for monomeric p7 have been proposed: a double membrane-spanning hairpin topology in the endoplasmic reticulum with the N- and C-termini facing the endoplasmic reticulum lumen (6), and an L-shaped form with the C-terminus facing the cytoplasm (7,8).

HCV p7 is capable of forming cation selective ion channels in planar lipid bilayers (9–12). In vitro HCV p7 ion channel activity can be inhibited by a range of compounds including amantadine, which also inhibits the viroporin M2 of the influenza A virus, and long alkyl chain iminosugar derivatives, such as *N*-nonyl-deoxynojirimycin (NN-DNJ), which are known to inhibit endoplasmic reticulum α glucosidase I and II as well as HCV p7 (11,13).

The oligomerization state of the channel has been under debate. Using electron microscopy, bacterially expressed GST-his-tagged and GST-FLAG-p7 monomers have been suggested to assemble into hexameric and heptameric ion channels, respectively (9,10). Molecular dynamics studies of the channel suggested a potential oligomerization of p7 monomers into a hexameric bundle, though other oligomerization states were not ruled out (14). Both the heptameric models of p7 proposed by Patargias et al. and Clarke et al. assume that the N-terminal helix of p7 lines the pore

(9,14). In the absence of high resolution structural data, it is desirable to establish which residues may line the pore to help interpret low resolution electron microscopy data.

Gandhi et al. showed that M2 can be inhibited by Cu^{2+} but not by Mg^{2+} (15). This inhibition is thought to be due to the chelation of Cu^{2+} by histidine side chains which line the pore of M2, thus causing ion-channel block. Analysis of the p7 sequence from 26 representative strains (Fig. S1 in the Supporting Material) indicates that histidines are found in the N-terminal helix of the p7 protein. One strain, H77, possesses only one histidine at position 17 (Fig. 1 A). We thus postulated that if H77-p7 channels were inhibited by Cu^{2+} , it would lend support to the hypothesis that the N-terminal helix lines the pore. Such information will be invaluable in the construction of future models.

Using chemically synthesized H77 p7 in black lipid membranes comprised of phosphatidylcholine and phosphatidylethanolamine lipids, we determined whether p7 ion channel activity could be inhibited by Cu^{2+} (see the Supporting Material for methods). Addition of 50 μM Cu^{2+} to the bathing solution reduces the relative mean current to ~20% (Fig. 1, B and C). The mean conductance drops from 25.5 ± 1.8 pS to 13.9 ± 1.2 pS over this time period, and the open channel probability, P_o , shifts from 0.33 ± 0.08 to 0.22 ± 0.05 . When the concentration of Cu^{2+} was increased to 500 μM , the inhibition was even more pronounced (mean conductance = 7.2 ± 0.5 pS, $P_o = 0.13 \pm 0.04$).

Control experiments with 1mM Mg^{2+} were performed (Fig. 1 D) where the channels were confirmed to be

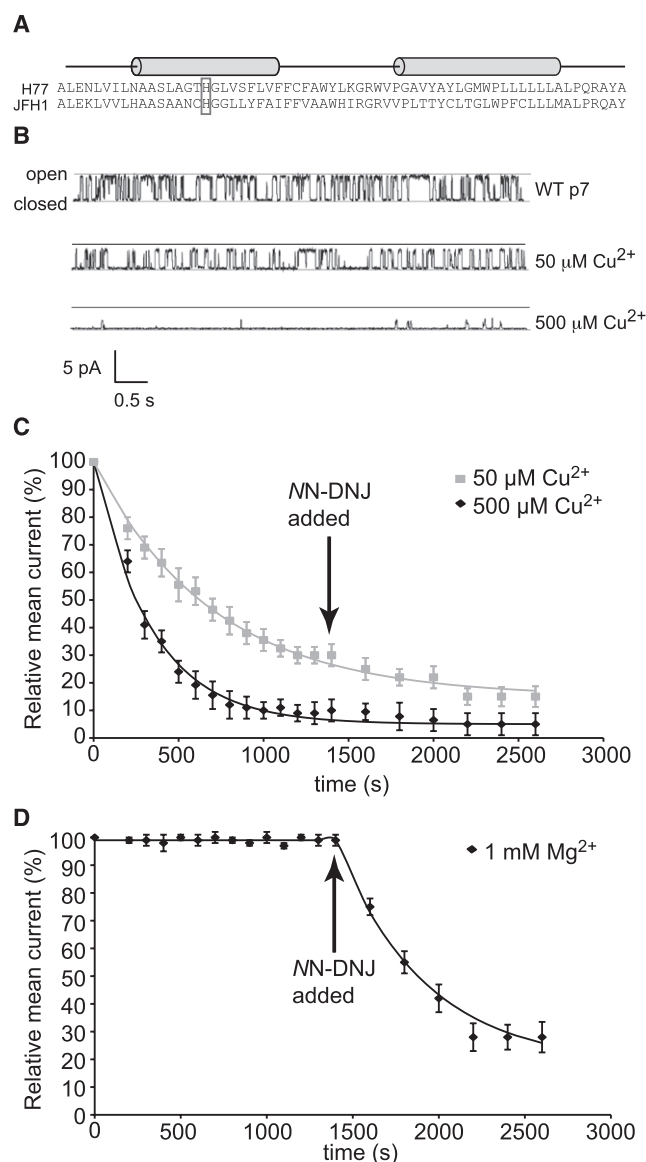


FIGURE 1 (A) Sequences of HCV p7 protein from the H77 and JFH-1 strains. The gray box indicates H-17. Cylinders indicate predicted helical regions. (B) Typical channel recording traces. (C) Time course of inhibition of the wild-type H77 p7 ion channel protein by 50 μM (gray squares) and 500 μM (black diamonds) Cu^{2+} . The symbols in this and the following figures show the recorded currents (mean \pm SE for $n = 10$), and the lines show the currents fit with an optimal exponential decay constant using MATLAB (The MathWorks, Natick, MA). (D) Time course of inhibition by 1 mM Mg^{2+} . At $t = 1400$ s, 500 μM of NN-DNJ was added to confirm the presence of p7 ion channels.

insensitive to Mg^{2+} (mean conductance 24.4 ± 1.6 pS, $P_o = 0.31 \pm 0.08$). Evidence that the channels were comprised of p7 protein was provided by the addition of 500 μM NN-DNJ at $t = 1400$ s, where Mg^{2+} treated channels were inhibited substantially (Fig. 1 D). NN-DNJ was also added to the experiments with Cu^{2+} at $t = 1400$ s, but in this case no further inhibition was observed because by this point the maximum level of inhibition had already been obtained.

Gandhi et al. showed that Cu^{2+} binds less strongly to His-37 of influenza M2 at low pH (5.2–6.2). Histidine residues in solution have a pK_a of ~ 6.5 . If H-17 is solvent exposed, we reasoned that it might show sensitivity to pH in terms of its protonation state and consequently its ability to coordinate Cu^{2+} . We examined the recovery from the Cu^{2+} induced block by addition of 0.05 M HCl into the *cis* chamber until the desired pH was reached. The results are summarized in Fig. 2 A and show that decreasing pH leads to an increase in the extent of recovery. We do not know the orientation of the channels within the black lipid membranes. One explanation of why the recovery is incomplete may be due to a proportion of the channels being oriented such that the H-17 is less accessible to protons in the *cis* chamber.

To test our hypothesis further, the mutant H-17A was synthesized and when reconstituted into a planar lipid bilayer, exhibited wild-type channel activity (mean conductance = 24.8 ± 1.7 pS, $P_o = 0.31 \pm 0.08$). In this case, however, the addition of 500 μM of Cu^{2+} to the bathing solution did not inhibit p7 mutant ion channel recordings but did inhibit wild-type p7, as seen in Fig. 2 B. As expected,

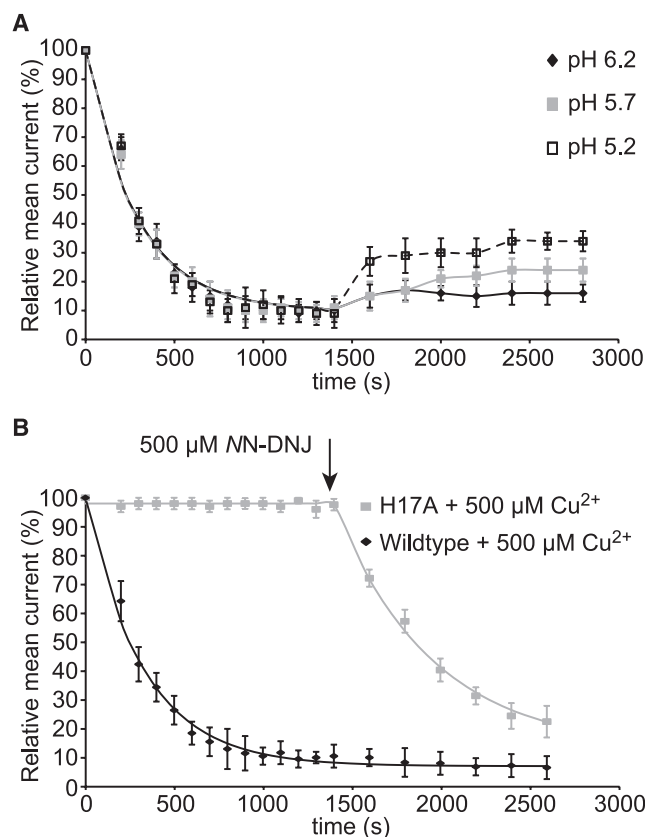


FIGURE 2 (A) Addition of 0.05 M HCl at $t = 1400$ s to pH 6.2 (black diamonds), pH 5.7 (gray squares), and pH 5.2 (open squares). (B) Comparison of the H-17A mutant peptide (gray squares) with wild-type p7 (black diamonds). 500 μM Cu^{2+} is added at $t = 0$. The addition of 500 μM NN-DNJ at $t = 1400$ s has little additional effect on the wild-type channel but inhibits the mutant channels.

the addition of 500 μ M NN-DNJ at 1400 s, inhibited both the wild-type and the mutant ion channel (Fig. 2 B).

Comparison of the H77 strain with the JFH-1 strain (Fig. 1 A) revealed that there is a histidine at an equivalent position. We reasoned that peptides from this strain should also exhibit Cu^{2+} inhibition, and the experiments described above were repeated for the JFH-1 strain. Results were very similar (Supporting Material), supporting our conclusions for the H77 strain.

We have demonstrated that Cu^{2+} is capable of inhibiting the p7 ion channel of HCV in a planar lipid bilayer. The inhibition is concentration-dependent. In addition, the effect of the inhibitor activity can also be recovered by decreasing the pH of the bathing solution. The lower the pH, the quicker and greater the reversal of inhibition appears to be. These studies lead us to conclude that the N-terminal helix of p7 lines the pore through which ions are conducted and that the H-17 residue faces the pore. This information should provide a useful constraint in the development of oligomeric models of the pore.

SUPPORTING MATERIAL

Methods, results, figures, and references are available at [http://www.biophysj.org/biophysj/supplemental/S0006-3495\(08\)00048-9](http://www.biophysj.org/biophysj/supplemental/S0006-3495(08)00048-9).

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