Structural characterisation of HC-Pro, a plant virus multifunctional protein

Célia Plisson¹, Martin Drucker², Stéphane Blanc², ⁴, Sylvie German-Retana³, Olivier Le Gall³,
Daniel Thomas¹, and Patrick Bron¹, ⁴

Célia Plisson and Martin Drucker contributed equally to this work.

Running title: Structural study of HC-Pro

¹ Université Rennes I, UMR 6026 CNRS, Campus de Beaulieu, 35042 Rennes, France
² Station de Recherches de Pathologie Comparée UMR 5087 INRA-CNRS-Université Montpellier II, 30380 Saint-Christol-lez-Alès, France
³ INRA, Virologie, IBVM, BP 81, 33883 Villenave d'Ornon Cedex, France

⁴ Corresponding authors: Patrick Bron, phone: +33 (0)2 23 23 69 82, fax: +33 (0)2 23 23 50 48, email: patrick.bron@univ-rennes1.fr and Stéphane Blanc, phone: +33 (0)4 66 78 37 15, fax: +33 (0)4 66 52 46 99, email: blanc@ensam.inra.fr
**SUMMARY**

The helper component proteinase (HC-Pro) is a key protein encoded by plant viruses of the genus *Potyvirus*. HC-Pro is involved in different steps of the viral cycle, aphid transmission, replication, virus cell-to-cell and systemic movement, and is a suppressor of post-transcriptional gene silencing. Structural knowledge of HC-Pro is required to better understand its multiple functions. To this aim, we purified his-tagged wild-type HC-Pro and a N-terminal deletion mutant (ΔHC-Pro) from plants infected with recombinant potyviruses. Biochemical analysis of the recombinant proteins confirmed that HC-Pro is a dimer in solution, that the N-terminus is not essential for self-interaction, and that a large C-terminal domain is highly resistant to proteolysis. Two-dimensional crystals of the recombinant proteins were successfully grown on Ni$^{2+}$-chelating lipid monolayers. Comparison of projection maps of negatively stained crystals revealed that HC-Pro is composed of two domains separated by a flexible constriction. Cryo-electron crystallography of ΔHC-Pro allowed to calculate a projection map at 9 Å resolution. Our data from electron microscopy, biochemical analysis, and secondary structure predictions, lead us to suggest a model for structure/function relationships in the HC-Pro protein.

**KEYWORDS**

HC-Pro/ protein structure/ 2D crystallisation/electron microscopy/ lipid monolayer/ biochemistry/ potyvirus
**INTRODUCTION**

The genus *Potyvirus* is one of the largest genera of plant RNA viruses. The potyvirus genome encodes a single polypeptide that is processed by three viral proteinases to release all viral proteins needed for the infection cycle. One of these proteins is HC-Pro (helper component proteinase). HC-Pro fulfils many functions in the viral cycle (reviewed by 1,2). It was first recognised as an indispensable helper factor for virus host-to-host transmission by aphid vectors (3). Later, a protease activity was found acting in cis on its own C-terminus to release it from the precursor polyprotein (4). Subsequent research identified further functions of HC-Pro: It is a general enhancer of infectivity and genome amplification, and is indispensable for cell-to-cell and systemic movement in the plant (5). More recently, it has been identified as a suppressor of post-transcriptional gene silencing (PTGS) and virus-induced gene silencing (VIGS) (6).

Mutagenesis studies and sequence alignments suggest that HC-Pro can be schematically divided into three regions: a N-terminal region essential for the transmission process, a C-terminal region harbouring the proteinase activity, and a central region implicated in all other functions (see Fig. 1). This view is probably too simplistic, and many functions most likely overlap in the HC-Pro sequence as described below.

The transmission function involves two conserved motifs. One is the N-terminal KITC motif [amino acid (AA) 52-55, numbering according to HC-Pro from *Lettuce mosaic virus* (LMV)] that is involved in binding to the aphid vector’s stylets (7). The other is the C-terminal PTK motif (AA 310-312) that probably contributes to binding of HC-Pro to the viral capsid protein's N-terminal DAG motif (8,9). The DAG motif/PTK motif interaction is essential for transmission, but a second HC-binding motif might exist in the capsid protein, perhaps with different functions (10). The N-terminal approximately 100 AA of HC-Pro seem
to be only involved in the transmission process because viral mutants deleted of this region are fully infectious (11).

The proteinase domain has been mapped to the C-terminal 155 AA and characterised as a cysteine protease-like activity with C$_{344}$ and H$_{417}$ residues in the active site (4). This region might also overlap with a cell-to-cell movement domain, as in *Bean common mosaic necrosis virus* HC-Pro, a C-terminal deletion of 87 AA and 293 AA, respectively, partially or totally abolished cell-to-cell movement of heterogeneously expressed protein in microinjection studies (12).

The central region of HC-Pro (AA 100-300) is generally assumed to be important in genome amplification (IGN motif, AA 260-262), synergism with other viruses, and systemic movement within the host plant (CC/SC motif, AA 292-295) (5,13). Two domains (A and B) spanning the entire central region, were described as non-specifically binding nucleic acids with a preference for ssRNA. The B domain shares homology to ribonucleoproteins (14,15).

Recently, HC-Pro has been recognised as a suppressor of PTGS and VIGS (for reviews see 16,17). PTGS, VIGS, and other RNA silencing phenomena are ancient defence mechanisms found in several eukaryotes and might constitute an alternative immune system where short interfering RNAs (siRNA) mediate specific degradation of aberrant RNA such as viral RNA. In plants, the phloem distributes an unidentified silencing signal throughout the plant and as a result, the whole plant suppresses expression of the concerned gene. Many plant viruses have evolved counterstrategies to knock out PTGS and encode PTGS suppressors such as HC-Pro. HC-Pro does not interfere with the mobile silencing signal but inhibits accumulation of siRNA through an unknown mechanism (18). The central region of HC-Pro is implicated in suppressor activity and overlaps with the region identified for genome amplification and viral movement (19).
Although many functions of HC-Pro have been well characterised by mutagenesis studies, little is known about the molecular mechanisms involved and the links between the various activities. Size exclusion chromatography suggests that the functional HC-Pro in transmission is a dimer or trimer (3,20). Yeast two hybrid experiments to test for HC-Pro self-interaction yielded somewhat confusing results. One group reported that only the N-terminus is involved in HC-Pro self-interaction (21), whereas another group identified interaction sites both in the N-terminus and the C-terminal proteinase domain (22).

Knowledge of the structure of HC-Pro will greatly contribute to understand its multifaceted functions and how structural domains are organised to fulfil them in concert or independently. To this aim, we analysed the structure of HC-Pro of LMV by biochemistry and electron microscopy. We fused a histidine tag (his-tag) to the N-terminus of both wild type HC-Pro and a 99 AA N-terminal deletion mutant (ΔHC-Pro) in the viral context. This strategy allowed purification of the recombinant proteins from infected plants and provided a convenient means for targeting HC-Pro to nickel-chelating lipid interfaces for electron microscopy of two-dimensional (2D) crystals (for a review, see 23). Biochemical analysis confirmed the dimeric nature of soluble HC-Pro and provided information on regions responsible for dimerisation. Furthermore, 2D crystals of the two recombinant proteins were obtained and suitable for structural analysis by electron microscopy and image processing. We present here projection maps of the two negatively stained recombinant proteins and a projection structure of frozen-hydrated ΔHC-Pro at 9 Å resolution. Finally we discuss relationships between HC-Pro’s structural organisation and biological functions.
EXPERIMENTAL PROCEDURES

Construction of recombinant viruses
Recombinant LMV-E mutants were obtained by insertion in the plasmid p70SLMVE of a (his)$_6$-tag fused either to the N-terminus of wild type HC-Pro or of a deleted form (99 AA) of HC-Pro. p70SLMVE contains an infectious, full-length cDNA copy of LMV-E RNA, under the control of the enhanced CaMV 35S promoter and the NOS terminator (24). The resulting recombinants were designated hisHC-Pro and hisΔHC-Pro. Briefly, we introduced a SmaI site (CCCGGG) at the level of the AatII site (nt 1418-1423) that corresponds to the second and third codons of the HC-Pro sequence. The resulting plasmid pLMV-E-SmaHC contains a new SmaI site surrounded by two AatII sites at the beginning of the HC-Pro coding sequence. Finally, the sequence CATCACCACCACCACCACAT encoding six histidines was inserted as a ds oligonucleotide in the newly created SmaI site. This yielded plasmid pLMV-E-hisHC-Pro where the viral protein P1 cleavage site necessary for liberation of HC-Pro from the polyprotein precursor is conserved. The AA sequence of the N-terminus of hisHC-Pro is SDVphhhhhhgdrvARN (inserted AA in italics). A similar strategy was used to clone pLMV-E-hisΔHC-Pro where the HC-Pro sequence was deleted of AA 4-102 in LMV-E-SmaHC, and where the histidine-tagged deleted HC-Pro has the following N-terminal AA: SDVphhhhhhgKQV. Sequencing of recombinant LMV hisΔHC-Pro RNA amplified by RT-PCR showed that the HC-Pro sequence of infectious hisΔHC-Pro virus recombinant contained a G to A substitution resulting in a G to R change of AA 183 (AA 285 in wild type HC-Pro) without any effect on infectivity of the virus.

Purification of HC-Pro and ΔHC-Pro
Young pea (Pisum sativum L.) plants were inoculated with the recombinant viruses as described (25). Pea leaves harbouring disease symptoms characteristic of either recombinant
virus were harvested two to three weeks after inoculation. They were homogenised in a blender together with 2 volumes of buffer ST (100 mM Tris, pH 8.0, 20 mM Mg$_2$SO$_4$, 0.5 mM EGTA) supplemented with 500 mM NaCl, 0.2 % Na$_2$SO$_3$, and 0.1 % PVP. The brei was filtered through four layers of cheesecloth and one layer of miracloth (Calbiochem), and centrifuged for 60 min at 100,000 g. To concentrate the supernatant containing HC-Pro, differential precipitation with (NH$_4$)$_2$SO$_4$ (20 - 40 % for hisHC-Pro and 20 - 50 % for hisΔHC-Pro) was carried out and the HC-Pro-containing fraction resuspended in ST buffer plus 0.5 M NaCl and stored at -70 °C.

Several preparations were thawed, centrifuged for 5 min at 5,000 g, the supernatant mixed with 1 ml Ni-NTA resin (Qiagen), and 10 % methanol was added. After 30 min incubation on ice, the resin was rinsed 2 times 30 min in a batch procedure with 50 ml ST buffer plus 500 mM NaCl and 10 % methanol, followed by 2 washes with 50 ml of ST buffer plus 100 mM NaCl. Then the resin was applied on a column and eluted with 5 ml ST buffer plus 100 mM NaCl and 500 mM imidazole. The elute was precipitated with 60 % (NH$_4$)$_2$SO$_4$, resuspended in ST buffer plus 1 M NaCl (2 M for hisΔHC-Pro) and loaded on a phenylagarose column (Sigma) operated at room temperature. The column was washed with five volumes of the same buffer and eluted with five volumes of ST buffer. The eluate was precipitated with 80 % (NH$_4$)$_2$SO$_4$, the pellet washed two times with 80 % (NH$_4$)$_2$SO$_4$ and the protein stored in 80 % (NH$_4$)$_2$SO$_4$ at 4 °C until use.

**Size exclusion chromatography**

Purified hisHC-Pro or hisΔHC-Pro was centrifuged at 10,000 g for 10 min before size exclusion chromatography with Ultrogel AcA 34 resin in a XK70 column (Pharmacia). The running buffer was ST buffer plus 100 mM NaCl. Gel runs were carried out on an ÄKTA Prime system (Pharmacia) at 4 °C with a flow rate of 0.1 ml/min and monitoring the
absorbance at 280 nm. The column was calibrated with a kit for molecular weights 29,000-700,000 Da (Sigma).

**Chemical cross-linking**

5 µl protein solution was incubated with 1 µl 12.5 mg/ml 1,8-bis-maleimidotriethyleneglycol (Pierce) solution in DMF or with 1 µl 0.1% glutaraldehyde for 5 - 60 min on ice. The reaction was quenched with 4x Laemmli buffer, the samples boiled for 5 min and the proteins analysed by SDS-PAGE. DMF alone had no effect on the oligomerisation state of the proteins.

**Limited proteolysis**

Solutions of hisHC-Pro or hisΔHC-Pro in buffer ST plus 100 mM NaCl were incubated for the times indicated with 1.0x trypsin solution (Gibco) at 37 °C. Adding 4x Laemmli buffer and boiling of the samples for 5 min stopped the reaction before analysis of the proteins by SDS-PAGE. For N-terminal sequencing of the 32-kDa proteolysis product, we employed identical conditions, except that 0.5 µg sequencing grade trypsin (Promega) was used for 1 µg of protein.

**N-terminal sequencing**

The proteolysis products were separated by SDS-PAGE and transferred to a PVDF membrane. The 32-kDa band was identified by Ponceau S staining, cut off, and extensively washed with water. N-terminal sequencing was carried out by Edman sequencing.

**2D crystallisation**

We obtained 2D crystals of hisHC-Pro and hisΔHC-Pro by the following method. A lipid mixture was made of the ligand lipid, DOGS-NTA-Ni, and the diluting lipid dioleoyl phosphatidylcholine (DOPC), at a molar ratio of 1:1 in chloroform/methanol (9/1 vol/vol) and a final concentration of 0.5 mg/ml. Lipids were purchased from Avanti Polar Lipids. 0.5 µl of the lipid mixture was spread on the surface of a drop in a Teflon well (54 µl) containing buffer A (20mM Tris-HCl pH 8.0, 200 mM MgCl₂). The Teflon wells used in these
experiments were designed for 2D crystallisation of membrane proteins on lipid layers (26). Each well was connected to a side hole allowing injection of the protein solution. After overnight incubation at room temperature in a humid chamber, 3 µl of protein solution was injected below the lipid layer and the solution gently stirred without perturbing the surface lipid layer. Crystallisation samples were incubated at room temperature in a humid chamber. After 24 to 48 h, a holey carbon grid was deposited on the surface of the drop, and the transferred sample negatively stained with 1% uranyl acetate for 30 seconds and then carbon-coated (27). For observation of frozen-hydrated 2D crystals of hisΔHC-Pro, plane carbon-coated gold grids were placed on top of the crystallisation wells and left in contact with the interface for two hours. Excess buffer was blotted with filter paper and the grids frozen rapidly in liquid ethane and stored in liquid nitrogen (28).

**Electron microscopy and image analysis**
Specimens were examined in a Philips CM12 electron microscope equipped with a LaB6 filament, operating at 120 kV. Suitable 2D crystals were imaged on Kodak SO-163 film at a precalibrated electron optical magnification of 43,750x, using low-dose techniques. Frozen-hydrated specimens were imaged using a Gatan 626 cryo-holder. Micrographs were developed for 12 min at 20 °C.

Micrographs selected by optical diffraction were digitised at 15 µm pixel size for negatively stained specimens and 10 µm for frozen-hydrated specimens with a HI-SCAN (Eurocore). Images were processed using the MRC image processing programs (29). Briefly, the Fourier transform of each image was calculated and the cell parameters determined. The images were corrected for distortions of the crystal lattice by cross-correlation Fourier analysis and re-interpolation of the image (30,31). Amplitudes and phases were extracted using MMBOXA. The defocus and astigmatism values were first determined by CTFFIND and subsequently refined during the merging procedure. Images were corrected for the
contrast transfer function using CTFAPPLY. Phase relationships between Fourier components of each crystal were examined with the program ALLSPACE (32) and the space group was determined. Comparison of the internal phase residual in all 17 space groups revealed $p4$ symmetry. The common origin was refined and the CTF-corrected data merged. The phases were rounded to either 0 or 180°, as the projection structure was centro-symmetric in this plane group. With the amplitudes and phases obtained after image processing, Fourier projection maps were calculated using the CCP4 program package (33).

**Sequence analysis and predictive methods**
Predictions of secondary structure of hisHC-Pro and hisΔHC-Pro were computed using the following programs: PHD (34,35), Hnn (35), nnpredict (36,37), Jpred (38,39) and SOPM_A (40).

**SDS-PAGE, Western blot, and antibodies**
SDS-PAGE was carried out using standard methods with 12% polyacrylamide gels. Gels were stained with Coomassie Blue or transferred to nitrocellulose using a semi-dry blotting apparatus. Bound antibodies were detected with the NBT/BCIP colour reaction or by enhanced chemiluminescence (Pierce). For detection of HC-Pro, a rabbit polyclonal antiserum was used, for detection of his-tags a commercial mouse monoclonal antibody (Eurogentec).
RESULTS

Expression of functional recombinant HC-Pro
Two LMV recombinants were constructed (Fig. 1), where a his-tag was inserted upstream of the wild type HC-Pro sequence (hisHC-Pro) or of a mutant deleted of AA 4 to 102 (hisΔHC-Pro). The recombinant viruses were used to inoculate young pea (Pisum sativum L.) plants. The two LMV mutants were fully infectious and plants displayed typical disease symptoms eight to ten days after inoculation. hisHC-Pro and hisΔHC-Pro were purified from infected plants as described in Materials and Methods. Fig. 2 shows that hisHC-Pro displayed a single band of the expected size of 53 kDa in SDS-PAGE whereas hisΔHC-Pro migrated consistently as a double band, one corresponding to the calculated mass of 41 kDa, the other being about 2 kDa smaller. In Western Blots probed with a anti-(his) subunit antibody, only the upper band was revealed, suggesting that hisΔHC-Pro is cleaved in planta at its N-terminus.

In aphid transmission tests, hisHC-Pro was active, whereas hisΔHC-Pro was inactive (data not shown). In protein overlay assays, the two HC-Pro variants interacted with the coat protein from several potyvirus species, in accordance with previous reports on HC-Pro from other potyviruses (8,9), further confirming structural integrity of the purified proteins.

The N-terminus of HC-Pro is not essential for oligomerisation
We subjected hisHC-Pro and hisΔHC-Pro to size exclusion chromatography to determine their native oligomerisation state (Fig. 3A). hisHC-Pro eluted at a volume equivalent to 138±35 kDa (4 independent experiments). This corresponds to a dimer or trimer, as already reported for HC-Pro from other potyviruses (3,20). Surprisingly, hisΔHC-Pro eluted at a volume equivalent to 96±11 kDa (3 independent experiments), also suggestive of a dimer or trimer. This indicates that the N-terminus of HC-Pro is not essential for oligomerisation. The results imply that soluble hisHC-Pro as well as hisΔHC-Pro are either globular trimers or non-
globular dimers. To determine more precisely the oligomeric state of soluble hisHC-Pro and hisΔHC-Pro, we subjected both proteins to chemical cross-linking with a SH-reactive compound, 1,8-bis-maleimidotriethyleneglycol. Fig. 3B shows that, after adding the cross-linker compound, both hisHC-Pro and hisΔHC-Pro migrated in SDS-PAGE as dimers, in addition to monomer forms. Occasionally, we observed also tetramers and higher oligomers. Experiments with lysine-reactive compounds and glutaraldehyde as cross-linking agents gave similar results (data not shown). In the case of hisΔHC-Pro, only one band corresponding to a dimer was visible instead of the three expected by random combination of the two subforms of hisΔHC-Pro. This might have been due to the poor resolution of the SDS-PAGE gels for high molecular mass proteins.

**HC-Pro contains a protease-resistant domain**
We carried out limited trypsic proteolysis of purified hisHC-Pro and hisΔHC-Pro. Fig. 4A shows that trypsin degraded hisHC-Pro to a 32-kDa peptide. This peptide was very resistant to prolonged proteolysis. Trypsin digestion of hisΔHC-Pro (Fig. 4A) also yielded a 32-kDa peptide. This indicated that the 32-kDa peptide originated from the middle or C-terminal part of the entire hisHC-Pro molecule. N-terminal sequencing of the 32-kDa peptides showed that both hisHC-Pro and hisΔHC were cleaved at AA T170 or G176 (numbering according to wild type HC-Pro) suggesting that this stretch of the HC-Pro molecule is exposed. Chemical cross-linking of the 32-kDa peptide of hisHC-Pro and hisΔHC with glutaraldehyde revealed that the region sufficient for dimerisation is contained in the C-terminal 282 AA of HC-Pro (Fig. 4B). Tetramers and higher oligomeric forms of HC-Pro were also observed as with full length HC-Pro and ΔHC-Pro. Chemical cross-linking experiments with SH-reactive and lysine-reactive compounds gave similar results (data not shown).
2D crystallisation of hisHC-Pro and hisΔHC-Pro.

2D crystallisation of hisHC-Pro and hisΔHC-Pro was achieved after binding onto a lipid monolayer. The nickel-chelating lipid used in this study was 1,2-dioleoyl-sn-glycero-3-[(N-(5-amino-1-carboxypentyl)imidodiacetic acid)succinyl] (nickel salt) (DOGS-NTA-Ni). A systematic screening of crystallisation conditions revealed that Mg²⁺ in the buffer was crucial to induce crystallisation of both recombinant proteins. hisHC-Pro crystals were extremely fragile and most often broke when transferred onto plain carbon grids. Subsequently, 2D crystals were transferred onto holey carbon grids and then carbon-coated (27).

Fig.s 5A and B show typical hisHC-Pro and hisΔHC-Pro 2D crystals. They had well-defined edges forming step-like structures. hisHC-Pro formed large 2D crystals with 1 to 10 µm in diameter containing many defaults when examined at higher magnification. Fig.s 5C and D show a representation of the Fourier components determined from a single image. The quality of hisΔHC-Pro crystals was clearly better than that of hisHC-Pro crystals. Indeed, for hisΔHC-Pro crystals, reliable factors up to 17Å resolution were visible in the optical diffraction pattern while the upper limit resolution obtained from hisHC-Pro crystals was usually comprised between 25 and 20 Å.

The unit cell parameters determined from averaged images are presented in Table 1. Although the molecular mass of hisΔHC-Pro is 22% smaller than that of hisHC-Pro, its unit cell is 12% larger. This certainly reflects different protein packing of hisΔHC-Pro in the crystal. Examination of the phase relationships indicated a p4 plane group for both 2D crystals (32).

Projection maps calculated from negatively stained 2D crystals reveal two structural domains

Images of crystals were recorded as described in Materials and Methods. An inherent problem of using holey carbon grids to pick up crystals at the interface of the crystallising wells is that most recorded images derive from more or less randomly tilted 2D crystals. Merging such
images leads to blurred projection maps. Thus, for a better understanding, we present projection maps calculated from single images. To better compare the projection maps, a resolution cut-off at 23 Å was applied to images of the two proteins before calculation of the final projection maps (Fig. 6).

The hisHC-Pro density map presented in Fig. 6A revealed a tetrameric organisation of the protein. The tetramer has a square-like shape with approximately 78 Å side length. The unit cell contains one tetramer and is presented by a black square box in Fig. 6A. The smaller repeat unit delimited in black shows an elongated form with a length of 54 Å, and is composed of two domains, designated 1 and 2, of approximately 26 Å and 22 Å in diameter, respectively. Domain 1 is located close to the centre of the tetramer and connected to domain 2 by a constriction. Four domains 2 form the corners of the tetramer. The hisHC-Pro monomer has been assigned to the domains 1 plus 2, based on comparison with projection maps of other soluble proteins (41,42) and taking into account the hypothetical surface hisHC-Pro would occupy with an overall bi-lobular shape.

The projection map calculated from negatively stained 2D crystals of hisΔHC-Pro was quite different and revealed an altered crystal packing (Fig. 6B). Two types of tetrameric motifs are observed in the projection map, located at the corner and in the centre of the unit cell, respectively. They overlap and show a mesh-like organisation (Fig. 6B). The smaller, elongated repeat unit encircled in black has a length of 57 Å and consists, like hisHC-Pro, of two globular domains of 26 Å and 22 Å in diameter, respectively. These domains were accordingly termed 1’ and 2’. Four domains 1’ form the tetrameric structure located at the corners of the unit cell whereas the tetrameric structure present into the centre of the unit cell results from merging of four domains 2’.

A comparison of the projection maps is presented in Fig. 7. The hisHC-Pro tetramer was not simply superimposable on the hisΔHC-Pro tetramer. Instead, a rotation of the entire
hisHC-Pro tetramer by $4^\circ$ was required to superimpose domains 1 and 1’. In addition, two further operations (rotation by $26^\circ$ and shift of 3 Å of domain 2) were necessary to superimpose domains 2 and 2’. These geometric operations suggest that the two domains are separated by a flexible region and are probably structurally independent. Structural modifications in hisΔHC induced more contacts between domains 2’, strengthening crystal stability and providing an explanation for the better quality of hisΔHC 2D crystals. Because hisHC-Pro and hisΔHC-Pro monomers were not directly superimposable, we computed average profiles of the two monomers to determine location of the domains in the HC-Pro sequence (Fig. 7B). Negative staining provides information about stain exclusion domains and a loss of density in a domain could be linked to a loss of volume. hisΔHC-Pro showed a density decrease in domain 1’, suggesting that the N-terminus is located in domain 1 of hisHC-Pro.

**Cryo-microscopy of hisΔHC-Pro**

2D crystals of hisΔHC-Pro being of higher quality than those of hisHC-Pro, they were used for further investigation by cryo-microscopy. Fig. 8A shows a typical computed transform with reliable reflections up to 8 Å resolution. The images clearly identify an tetragonal unit cell with $a = b = 103$ Å. Examination of the phase relationships indicated a $p4$ plane group (32) as found for negatively stained crystals. Four images were analysed and combined to generate a data set of 101 averaged reflections up to 8.6 Å resolution. After refinement to 0° or 180°, the overall phase residual up to 8.6 Å resolution was 24.6° (45° would be random), indicating that all symmetry-related reflections were reliable (Table 1). A fully symmetrised projection map at 9 Å resolution is presented in Fig. 8B. In this projection structure, the molecular boundaries of the hisΔHC-Pro monomer are now more clearly resolved and delimited in black in Fig. 8B. hisΔHC-Pro has a length of 63 Å and is composed of two domains as in the negatively stained projection map. Domain 1’ has a square-like shape with
27 Å side length, and is connected to domain 2’ by a constriction of roughly 16 Å diameter. Domain 2’ has a rectangular shape with 27 Å in length and 22 Å in width. Variation in measurements with those obtained from negatively stained crystal projection maps likely resulted from the stain drying process. The map reflects the projected electron density of the protein and subsequently is directly related to its mass. Thus, the whole density of hisΔHC-Pro corresponds to a molecular mass of 41,325 Da. Domains 1’ and 2’ represent 63% and 37% of the density, corresponding to masses of about 26 kDa and 15 kDa and consecutively to about 228 and 139 AA. New structural features are clearly resolved in the projection map, in particular high density peaks are seen in domains 1’ and 2’. These peaks have a round shape of 10 to 15 Å in diameter and might be related to projection of α helices (42,43).

**Predictions of the secondary structures suggest two structural domains**

Prediction of the secondary structure of hisHC-Pro was carried out using different programs. All these programs gave similar results. Fig. 9A presents the results obtained with the Hnn program (35). Two helix-rich regions (AA 40-235 and AA 330-458) connected by a less structured region of about 95 AA are predicted (Fig. 9B). This suggests that HC-Pro is composed of two compact domains connected by a less structured domain. This assumption correlates strongly with our experimental results, notably with the projection map obtained from frozen-hydrated hisΔHC-Pro 2D crystals. We showed that the N-terminus is located in the larger domain of HC-Pro, i.e. domain 1. This correlates well with the first predicted helix-rich domain located at the N-terminal extremity. We conclude that the HC-Pro structure determined here is closely related to the predicted secondary structure, the two helix-rich regions and the less structured region corresponding to structural domains 1 and 2 and the hinge domain, respectively.
DISCUSSION

The main goal of this study was to provide information on structure of the potyviral multifunctional protein HC-Pro in order to relate structure with biological functions. Three-dimensional crystallisation trials not being successful, we decided to elucidate the structure of HC-Pro by biochemistry, electron microscopy, and image analysis.

Earlier work by Thornbury et al. (1985) and Wang and Pirone (1999) demonstrated that the HC-Pro of Tobacco vein mottling virus, Potato virus Y, and Turnip mosaic virus behave like dimers or trimers in size exclusion chromatography. We confirmed these data for the LMV hisHC-Pro and suggest that this could be a general feature of potyviral HC-Pro molecules. We were unable to discern the exact oligomerisation state of soluble HC-Pro from our chromatography results. However, in chemical cross-linking experiments, hisHC-Pro reaction products appeared with a mass corresponding to a dimer but never to a trimer. Our data thus strongly suggest that soluble HC-Pro is a dimer and that the aberrant elution behaviour of HC-Pro in gel filtration is probably caused by its elongated shape. Structural characterisation of HC-Pro by electron microscopy confirms these assumptions.

We successfully grew 2D crystals of the two recombinant proteins on lipid monolayers containing Ni-NTA (23,44). Crystallisation occurred only in the presence of Mg$^{2+}$. This cation has earlier been shown to increase potyviral transmission rate in in vitro assays where aphids were fed crude or partially purified HC-Pro-containing extracts from infected plants (45). Our own observation reported here possibly indicates that Mg$^{2+}$ has an unknown organising and/or stabilising effect on the HC-Pro molecule.

Both recombinant proteins crystallised with $p4$ symmetry. It is quite surprising to obtain a $p4$ plane group symmetry since we prior demonstrate that HC-Pro has a dimeric behaviour in solution. Our observations (P. Bron, unpublished data) suggest that hisHC-Pro and hisΔHC-Pro first interacted with the lipid monolayer as dimers and then started to form
dimers of dimers resulting in a tetrameric state. Unfortunately, at this stage, we can not conclude on the structural organisation of the HC-Pro dimer in solution.

The analysis performed with negatively stained 2D crystals showed that both hisHC-Pro and hisΔHC-Pro have an elongated shape and are composed of two structural domains. Comparison of projection maps allowed us to conclude that the two structural domains are connected by a flexible region and that the N-terminus is located in domain 1. The fact that domains 1 and 1’ of the two proteins have equivalent diameters suggests that the domain formed by the first 102 N-terminal AA in hisHC-Pro is located in the perpendicular plane of the crystal. Although lacking 22% of the mass of the wild type protein, hisΔHC-Pro is still organised as a tetramer in the crystal. These observations suggest that the N-terminal 102 AA do not interfere strongly with the rest of the molecule and are likely structurally isolated. This could be confirmed by comparing three-dimensional models of hisHC-Pro and hisΔHC-Pro.

Several reports (21,22,46) suggested that the cysteine-rich region located in the N-terminal region of HC-Pro might contain a self-interaction domain due to its homology with Zn-finger-like motifs (47). While we do not rule out that this region is capable of self-interaction or interaction with other molecules, we present here convincing evidence that this region is not essential for dimerisation or functions other than transmission. First, symptoms caused by the LMV mutant harbouring hisΔHC-Pro were indistinguishable from those induced by the wild type virus, indicating that all functions required for infection are located downstream of the deletion. Second, properties of hisΔHC-Pro in size exclusion chromatography, cross-linking, and crystallisation were very similar to hisHC-Pro. The hisHC-Pro projection map shows that the tetrameric organisation results from interactions of domains 1, which contain the N-terminal extremity. However, intermolecular contacts between domain 2 and domain 1 were also observed. In hisΔHC-Pro projection structures, the tetrameric organisation is conserved and results from similar interactions. The only possible
conclusion of these results is that the N-terminal 102 AA of HC-Pro are not essential for dimerisation and that an essential self-interaction domain is located in the remaining part of the molecule. Finally, cross-linking studies with a 32-kDa trypsin digestion product of hisHC-Pro further delineated a dimerisation domain to the C-terminal 282 AA. This is consistent with the results from Guo et al. (1999) who identified a HC-Pro self-interaction domain in the C-terminal ~130 AA of HC-Pro by yeast double hybrid assay.

Predictions of the secondary structures of HC-Pro are consistent with our results, notably with the projection structure at 9 Å resolution of frozen-hydrated hisΔHC-Pro. This suggests a linear correlation between structural domains identified in projection maps and domains predicted from the amino acid sequence analysis (Fig. 9). Combined together, the results enable us to propose a structure/function model for HC-Pro. HC-Pro is elongated and composed of different structural domains. We propose that the proteinase activity previously mapped to the C-terminal 155 AA (4) corresponds to structural domain 2'. The structural domain 1, rich in α-helices, contains the hinge at its C-terminal extremity. The hinge region, suggested by secondary structure prediction and identified in the projection maps as a constriction between domains 1 and 2, was remarkably resistant to trypsin digestion, implying that it is probably well structured. Prediction programs indicate that this region is composed mainly of β-sheets. This structural organisation does not rule out the small dislocation of the two domains as observed from comparison of hisHC-Pro and hisΔHC-Pro projection maps. The ~90 AA comprising this region overlap well with RNA binding domain B (15) and contains important conserved motifs like IGN, CC/SC, and PTK. Thus, this section of HC-Pro is most probably more than just a hinge but presents a domain of its own. Functionally, it is associated with virus movement, genome amplification, RNA silencing, and coat protein binding. The N-terminal extremity of the structural domain 1’ corresponds to the helix-rich region 1 minus the first 99 AA. The region comprising AA 170-176 is probably exposed and not structured. This is shown by susceptibility of this region to trypsin digestion, resulting in
fast degradation of the entire N-terminus. It is interesting to note that the highly conserved FRNK motif is just downstream of AA 176. Likely, structure or location shielded this box from trypsin digestion because it was not degraded although it contains potential trypsin cleavage sites. This part (AA 100-225) of helix-rich region 1 corresponds to the previously identified RNA binding site A (15) and functionally contains parts of the regions involved in viral movement, genome amplification, and RNA silencing. As mentioned above, although helix-rich region 1 stretches from AA 1 to 275, the first 102 AA should be structurally isolated from the rest of the molecule since its deletion does not modify the projection structure of the molecule in negative staining.

In conclusion, we establish here the first basis to relate structure of HC-Pro with its multifunctional roles. It is interesting to observe that all functions of HC-Pro, except the self-cleavage function that is fully contained in domain 2, involve more than one structural domain. The transmission function needs the N-terminus and the PTK motif in the hinge domain. RNA silencing, virus movement, and genome amplification are associated with domain 1 and the hinge domain. The latter functions were suggested to be closely related (19). Whether this implies that they use the same active sites in domain 1 and the hinge, that different sites or combinations of sites in the same region are used, or that different host or viral interaction partners compete with the same sites for different functions, remains to be determined. We here hypothesise that domain 1 contains the active sites needed for the various functions and that the hinge domain regulates their accessibility by moving domain 2 to mask or expose domain 1. The movement of the hinge domain could, in turn, be regulated by interaction with various host or viral partners.
ACKNOWLEDGEMENTS

We thank Denis Chretien, Cyrille Garnier, and Isabelle Arnal for their critical reading of the manuscript. Thanks to Hervé LeCoq for HC-Pro antiserum, Pascal Espérandieu for gardening, and to Jean Derancourt for help with protein sequencing. The work was partly supported by a grant from the M.E.S.R. to C.P.
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LEGENDS TO FIGURES

Figure 1: Functional regions of HC-Pro and recombinant HC-Pro used in this study. (A) HC-Pro can be nominally divided into three regions. Numbering of amino acids (AA) is according to the HC-Pro sequence of LMV-E (B) Location of the putative vector-binding, RNA-binding, and viral capsid-binding regions in the HC-Pro sequence. Zn = putative zinc finger; A, B = RNA-binding domains. (C) Biological functions of different HC-Pro regions and position of conserved motifs. The arrow indicates that the PTK motif is also involved in transmission. (D) Recombinant HC-Pro mutants used in this study. A (his)$_6$-tag was fused adjacent of the N-terminal cleavage site of full length (hisHC-Pro) or deleted (AA 4-102) HC-Pro (hisΔHC-Pro).

Figure 2: Purification of hisHC-Pro and hisΔHC-Pro. (A) Coomassie Blue-stained SDS-PAGE gel of purified hisHC-Pro and hisΔHC-Pro. (B) Western blot of purified hisHC-Pro and hisΔHC-Pro probed with a polyclonal HC-Pro antiserum. (C) Western blot of purified hisHC-Pro and hisΔHC-Pro probed with a monoclonal (his)$_6$ antibody. Lanes 1 correspond to hisHC-Pro, lanes 2 to hisΔHC-Pro. Molecular mass markers are indicated at the left.

Figure 3: hisHC-Pro and hisΔHC-Pro are dimers. (A) Elution profiles of hisHC-Pro (1) and hisΔHC-Pro (2) in size exclusion chromatography. The elution peak of hisHC-Pro corresponds to a molecular mass of 138 kDa, that of ΔHC-Pro to a mass of 96 kDa. The inserts show a Coomassie Blue-stained SDS-PAGE gel of the peak fractions containing hisHC-Pro (1) and a Western Blot of the fractions containing hisΔHC-Pro (2). Elution peaks of molecular mass markers are indicated on the x-axis. (B) Chemical cross-linking with 1,8-
bis-maleimidotriethyleneglycol gives rise to a reaction product corresponding to a dimer of hisHC-Pro (left panel) and hisΔHC-Pro (right panel). Purified protein solutions were (lanes +) or were not (lanes -) subjected to cross-linking and analysed by Coomassie Blue staining after SDS-PAGE.

Figure 4: **hisHC-Pro and hisΔHC-Pro contain a trypsin-resistant dimerisation domain.** (A) Purified hisHC-Pro (first two lanes) and hisΔHC-Pro (next two lanes) were incubated with trypsin for the times indicated and digestion products analysed by Coomassie Blue staining after SDS-PAGE. The 32-kDa peptide (arrowhead) was readily detectable even after prolonged digestion (not shown). (B) The 32-kDa peptide released by trypsin digestion is cross-linked by glutaraldehyde to a dimer. The panels show Coomassie Blue-stained gels after SDS-PAGE of trypsin digests of hisHC-Pro (left panel) and hisΔHC-Pro (right panel) that were (lanes +) or were not (lanes -) subjected to cross-linking after trypsin digestion. Note that additional cross-linked products corresponding to a tetramer of the 32-kDa peptide were visible.

Figure 5: **Two-dimensional crystals of hisHC-Pro and hisΔHC-Pro.** Electron micrographs of negatively stained 2D crystals of hisHC-Pro (A) and hisΔHC-Pro (B) were recorded using a Philips CM12 electron microscope at a magnification of 43,750×. 2D crystals grew on lipid monolayers consisting of Ni-NTA DOGS/DOPC at a molar ratio of 1/1. Scale bars correspond to 100 nm. (C) and (D) show the Fourier components calculated from images A and B. Concentric lines indicate the zero values of the phase-contrast transfer function for a defocus of 8726 Å and 14473 Å, respectively. H and K axes refer to the reciprocal lattice vectors. Each box represents a reflection, and the size and the number within it (IQ values) relate to the quality of the data. A low IQ value reflects a high signal-to-noise ratio.
Black arrows in C and D indicate a resolution of 21.7 Å and 14.6 Å, respectively.

**Figure 6: Projection map of negative-stained 2D crystals of hisHC-Pro and hisΔHC-Pro.**
(A) Projection map of hisHC-Pro. A hisHC-Pro monomer, encircled in black, is composed of two structural domains named 1 and 2, of 26 Å and 22 Å in diameter, respectively. Total length of the monomer is 54 Å. (B) Projection map of hisΔHC-Pro. The crystal packing involves more intermolecular contacts. The monomer is composed of two structural domains labelled 1’ and 2’ with 26 Å and 22 Å in diameter. hisΔHC-Pro is 57 Å long. Four adjacent domains 2’ form the tetramer present in the middle of the unit cell. Maps were calculated from a single image and none symmetry operation was imposed. A resolution cut off of 23 Å was applied before computing the maps. The unit cells are presented by a black square box; for hisHC-Pro a = 96 Å, b = 95 Å, γ = 90°, and hisΔHC-Pro a = 102 Å, b = 100 Å, γ = 89°.

**Figure 7: Comparison of projection maps from negative-stained 2D crystals**
(A). The hisHC-Pro and hisΔHC-Pro tetramers shown were extracted from projection maps of negatively stained crystals. The middle inset displays superposition of hisΔHC-Pro (in blue) and hisHC-Pro. A rotation of 4° of the whole hisΔHC-Pro tetramer is required to superimpose domains 1 and 1’. Two additional geometric operations are needed to superimpose domains 2 and 2’, a rotation of 26° plus a shift of 3 Å.
(B). hisHC-Pro and hisΔHC-Pro averaged profiles. Profiles were normalised in order to be comparable. “1” and “2” above the curves refer to structural domains. The 99 AA deletion in hisΔHC-Pro results in a density decrease in domain 1’.
**Figure 8: Projection structure of frozen-hydrated 2D crystals of hisΔHC-Pro.** (A) Fourier components calculated from a single image of a hisΔHC-Pro 2D crystal. The edge of the plot corresponds to a resolution of 5 Å. The axes refer to the reciprocal lattice vectors. (B) Projection map of hisΔHC-Pro. The projection density map with p4 symmetry imposed was calculated to 9 Å resolution using amplitudes and phases from four images. One unit cell with a = 103 Å, b = 103 Å and a hisΔHC-Pro monomer are outlined in black. The zero-level was derived from the mean density of the map.

**Figure 9: Alignment of structural domains, predicted helices, and biological functions of HC-Pro.** (A) HC-Pro secondary structure as predicted by the Hnn program. Black boxes represent regions with high α-helix probability, open boxes regions with high β-sheet probability. (B) Schematic drawing of the regions covering the suggested two helix-rich regions that are separated by a less structured hinge region. Limits of these domains correspond to the average limits obtained from the different predictive methods. The arrow indicates the trypsin cleavage sites at AA 170 and 176. (C) Position of structural domains 1’ and 2’ of hisΔHC-Pro with the assumption that structural domains and sequence are linearly correlated. (D) and (E) same as in Fig. 1.
Table 1. Crystallographic data

### NEGATIVE STAIN

| Plane group symmetry | HCpro | ΔHCpro |
|----------------------|-------|--------|
| P4                   | P4    |        |
| Unit cell parameters (Å) |       |        |
| a= 95.6 ± 0.6 Å     | a= 101.9 ± 1.1 Å |
| b= 94.5 ± 0.9 Å     | b= 99.5 ± 2.2 Å |
| γ= 90.3 ± 1.4°      | γ= 89.3 ± 2.5° |

### CRYO

| Plane group symmetry | ΔHCpro |
|----------------------|--------|
| P4                   |        |
| Unit cell parameters (Å) |        |
| a= 103.0 ± 0.2 Å     |        |
| b= 102.9 ± 0.3 Å     |        |
| γ= 90.0 ± 0.3°       |        |

| Resolution range in Å | Number of unique reflection | Phase residual (deg.) compared to 0°/180° with IQ 5 max. (45° is random) |
|-----------------------|----------------------------|--------------------------------------------------------------------------|
| 300.0 - 17.4          | 28                         | 17.7                                                                     |
| 17.4 - 12.2           | 26                         | 23.4                                                                     |
| 12.2 - 10.0           | 27                         | 25.3                                                                     |
| 10.0 - 8.6            | 20                         | 34.9                                                                     |
| Total range           | 101                        | 24.6                                                                     |
Fig 1.
Fig 3.
Fig 4.
Fig 5.
Fig 6.
Fig 7.
Fig 9.
Structural characterisation of HC-Pro, a plant virus multifunctional protein
Célia Plisson, Martin Drucker, Stéphane Blanc, Sylvie German-Retana, Olivier Le Gall,
Daniel Thomas and Patrick Bron

J. Biol. Chem. published online April 8, 2003

Access the most updated version of this article at doi: 10.1074/jbc.M302512200

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