Site-directed mutagenesis of a potyvirus coat protein and its assembly in *Escherichia coli*

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Multiple copies of the Johnsongrass mosaic virus coat protein synthesized in *Escherichia coli* can readily assemble to form potyvirus-like particles. This *E. coli* expression system has been used to identify some of the key amino acid residues, within the core region of the coat protein, required for assembly. The two charged residues R\textsuperscript{194} and D\textsuperscript{238} previously proposed theoretically to be involved as a pair in the construction of a salt bridge crucial for the assembly process were targeted for site-directed mutagenesis. The results from our experiments suggest that the two residues are required for the assembly process but are not necessarily involved as a pair in a common salt bridge.

Potyviruses are flexuous, rod-shaped particles 700 to 900 nm long and 11 nm wide (Hollings & Brunt, 1981), containing a single copy of a positive sense, single-stranded, polyadenylated RNA of about 10 kb. The capsid of a mature virus particle consists of about 2000 copies of a single type of coat protein monomer arranged in a helical manner around the viral RNA (Shukla & Ward, 1989). The *M*<sub>s</sub>s of the coat protein of various potyviruses are in the range of 28K to 40K with the majority being in the 33K to 34K range.

Wild-type potyvirus particles can be dissociated and the coat protein monomers reassembled *in vitro* to form long flexuous potyvirus-like particles in the presence or absence of RNA (McDonald & Bancroft, 1977). The dissociated coat protein has been shown to polymerize to form stacked rings, which then assemble further into filamentous rods of 10·5 to 11 nm in diameter and of varying lengths up to several \(\mu\)m. Potyvirus particles mildly treated with trypsin, which removes the surface-exposed N and C termini, or lysyl endopeptidase, which removes only the N-terminal region of most potyvirus coat proteins (Shukla *et al.*, 1988), can also be dissociated and reassociated into potyvirus-like particles (Jagadish *et al.*, 1993) indicating that all the necessary information required for polymerization of the coat protein is located within its core region.

To date very little is known about the three-dimensional structure of the potyvirus particle or coat protein monomer and the mechanism of assembly. We have used site-directed mutagenesis followed by expression of a modified Johnsongrass mosaic virus coat protein gene in *Escherichia coli* and electron microscopic analysis, to initiate identification of some of the residues within the core region crucial to self-polymerization. We have previously shown that complete coat protein synthesized in the yeast *Saccharomyces cerevisiae*, or with an additional 16 amino acids at its N terminus in *E. coli*, readily assembled to form potyvirus-like particles (Jagadish *et al.*, 1991). These particles were similar in their morphology to those assembled *in vitro* in the absence of viral RNA (McDonald & Bancroft, 1977). We showed that mutagenic changes of the highly conserved residues RQ at positions 194/195 to DL abolished particle formation even though the levels of the mutant coat protein produced in *E. coli* were high. Dolja *et al.* (1991) have observed that R\textsuperscript{194} is highly conserved not only in the coat proteins of potyviruses but also in those of other flexuous plant viruses, and have suggested that it might be involved in the construction of a salt bridge with D\textsuperscript{238} which is present in the highly conserved AFDF sequence located towards the C-terminal region. In this report we have introduced a series of single amino acid changes at both R\textsuperscript{194} and D\textsuperscript{238} to test this suggestion.

In the first round of mutagenesis, the following non-conservative mutational changes were made to the coat protein. Initially, a derivative of the previously described mutant pTTQ19:CP\textsuperscript{RQ-Q} (in which R\textsuperscript{194}Q\textsuperscript{195} have been changed to DL) was made to change D\textsuperscript{238} to R resulting in a triple mutant construct, pTTQ19:CP\textsuperscript{RQ-DL/D238R} (Fig. 1). This was achieved by using ssDNA of the phagemid pT3T718U:CP\textsuperscript{RQ-Q} (Jagadish *et al.*, 1991) and the Dut−Ung site-directed mutagenesis method (Kunkel *et al.*, 1987). The newly introduced changes were confirmed by DNA sequencing and the mutagenized
fragment was cloned into pTTQ19. In the mutant pTTQ19:CP<sub>R194K</sub> (Fig. 1), only the residue R<sup>194</sup> was changed to D in contrast to the previously described double mutant pTTQ19:CP<sup>R194K</sup> (Jagadish <i>et al.</i>, 1991) where both R<sup>194</sup> and Q<sup>195</sup> were mutated. This was done to eliminate any effects, in the new mutant pTTQ19:CP<sup>R194K</sup>, that might have been caused by the introduction of the amino acid residue L in place of Q<sup>195</sup> adjacent to R<sup>194</sup>. In pTTQ19:CP<sup>D238E</sup>, only D<sup>238</sup> was changed to R; in the double mutant pTTQ19:CP<sup>R194K/D238R</sup>, R<sup>194</sup> and D<sup>238</sup> were changed to D<sup>194</sup> and R<sup>238</sup> respectively (Fig. 1).

To obtain the above mutational changes, PCR procedures were employed using SCMBI-1 phagemid DNA (Jagadish <i>et al.</i>, 1991), or DNA from one of the existing constructs, as a template and appropriate primers designed to include BamHI–NsiI restriction enzyme recognition sequences and the desired mutations. The PCR products were purified, restricted with BamHI and NsiI and were used to replace the corresponding fragments in the construct pTTQ19:CP or the mutant construct pTTQ19:CP<sup>R94Q-DL/D238R</sup> to obtain the desired combination of amino acid changes as shown in Fig. 1. The changes were confirmed by ssDNA or dsDNA sequencing. The general procedures for the use of recombinant DNA technology were as described (Sambrook <i>et al.</i>, 1989). The mutant constructs were transformed into E. coli strain DH1 and expressed by IPTG induction as described before (Jagadish <i>et al.</i>, 1991).

The cell extracts were analysed by SDS-PAGE/Western blotting methods. The levels of synthesis as well as the Mrs of the predominant bands that reacted with a polyclonal antiserum (JG: Core AS), raised against the core portion of the coat protein, were similar to those of the pTTQ19:CP product without any mutagenic changes in the coat protein-coding region (data not shown). However, direct or immune electron microscopic analysis of the E. coli extracts expressing the mutant constructs did not reveal the presence of potyvirus-like particles. The absence of particle formation, even in the double mutant in which the complementary charges of the two residues swapped have been maintained, suggests that other secondary effects may have interfered in coat protein assembly or that these two residues may not interact to form a common salt bridge.

In a new round of mutagenesis more conservative changes were introduced by using Kunkel’s site-directed mutagenesis method. In the mutant pTTQ19:CP<sup>D238E</sup>, D<sup>238</sup> was changed to E by using a single mutagenic primer and SCMBI-1 template DNA; in the mutant pTTQ19:CP<sup>R194K</sup>, R<sup>194</sup> was changed to K; in the double mutant pTTQ19:CP<sup>R194K/D238R</sup>, both R<sup>194</sup> and D<sup>238</sup> were changed to K and E respectively, using both mutagenic primers and SCMBI-1 template DNA. The mutagenized DNAs were excised as BamHI–BalI fragments and used to replace the corresponding regions in pTTQ19:CP. The new mutant constructs were expressed in E. coli DH1. As observed before (Jagadish <i>et al.</i>, 1991) and consistent with other mutant constructs described in this report, there were two large predominant bands that reacted in Western blots with JG: Core AS (Fig. 2). The smaller of these polypeptides which is similar in size to the coat protein (34K) could be the result of either internal initiation at the introduced methionine codon at the start of the coat protein-coding region (Jagadish <i>et al.</i>, 1991) or proteolytic cleavage of the larger protein. The slightly larger one could be due to the presence of the additional 16 amino acids at the N terminus (Jagadish <i>et al.</i>, 1991). There were also much smaller proteins present in all extracts; these could have been generated by degradation of larger coat protein molecules. However, the mobility pattern of smaller bands from extracts of E. coli that synthesized coat protein capable of forming potyvirus-like particles (lanes 2 and 4) is slightly different to the bands from extracts of E. coli that synthesized mutant coat protein incapable of forming such structures (lanes 3 and 5), indicating variations in protein folding. The smaller proteins may also have been generated owing to internal initiation or premature termination of translation.
Short communication

Fig. 2. Immunoblot analysis of mutagenized coat protein synthesized in E. coli and probed with the polyclonal antiserum JG:Core AS raised against purified, denatured coat protein of Johnsongrass mosaic virus particles. The bands were revealed using the alkaline phosphatase reaction. Lane 1, pre-stained M, standards (BRL) and a sample of purified virus mixed before loading; lane 2, E. coli DH1/pTTQ19:CP; lane 3, DH1/pTTQ19:CPD238E; lane 4, DH1/pTTQ19:CPR194K; lane 5, DH1/pTTQ19:CPR194K/D238E.

Immune electron microscopic analysis of cell extracts showed particles occurring only in cells expressing the mutant construct pTTQ19:CPR194K. These particles were similar in number and morphology to those found in cells expressing the construct pTTQ19:CP (Fig. 3a and b). In several experiments involving extracts of E. coli induced to express pTTQ19:CPD238E or pTTQ19R194K/D238E, immune electron microscopy failed to reveal structures with any definite morphology, implying unassembled coat protein molecules. The absence of assembly in cells expressing the double mutant pTTQ19:CPR194K/D238E suggests that the two residues do not necessarily pair to form a common salt bridge. It is more likely that R194 and D238 pair with other charged residues within the core region of the same subunit or neighbouring subunits. The lack of assembly of the mutant pTTQ19:CPD238E coat protein despite preservation of the negative charge may be due to other distortions caused by the bulkier E residue. In contrast, the substitution of R194 with the smaller K residue had no deleterious effect on particle formation. Although K was an acceptable replacement for R at position 194 for coat protein assembly in E. coli, no such change at this position was found in the coat protein sequences of 66 strains of 26 members of the Potyviridae. Similarly, none of the tobacco mosaic virus (TMV) strains sequenced to date have K or D as substitutions for R63 and E145 respectively, the two residues demonstrated by crystallographic studies to form a salt bridge. Thus it is possible that the constraints of protein structure may not readily permit the replacement of a residue involved in a salt bridge even with one of the same charge.

On the basis of predictions of secondary structure, and the surface location of the N- and C-terminal regions of the coat protein, it has been suggested that all rod-shaped viruses may share structural features with TMV (Shukla et al., 1988; Shukla & Ward, 1989) whose three-dimensional structure is known. The three-dimensional structure of TMV (Bloomer et al., 1978) shows that the central part of the molecule consists of a group of four alpha-helices which are arranged closely parallel or antiparallel to each other. These helices are held together on the distal surface by a short four-stranded anti-parallel beta-sheet and by intra- and inter-subunit salt bridges involving several conserved R, K, E and D residues (Bloomer et al., 1978). The salt bridge between the conserved R63 and E145 discussed by Dolja et al. (1991)
occurs in this distal beta-sheet region of the TMV coat protein monomer, as an intra-subunit connection (Altschuh et al., 1987). In the predicted model of the potyvirus coat protein (Shukla et al., 1988) the positions of the very highly conserved R<sub>194</sub> and D<sub>238</sub> residues are not equivalent to the R<sub>61</sub> and E<sub>145</sub> residues of TMV coat protein. The residue R<sub>194</sub> is predicted to reside within one of the alpha-helices and D<sub>238</sub> is located near the centre of the virus particle, moreover the two residues are considerably closer (separated by only 43 residues) than R<sub>61</sub> and E<sub>145</sub> of TMV. The results from our site-directed mutagenesis experiments show that although the two residues R<sub>194</sub> and D<sub>238</sub> are crucial for potyvirus coat protein structure and assembly, they may not necessarily form a common salt bridge. A more definitive answer can only be obtained by a three dimension-structural analysis of the virus.

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References


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