Analysis of the role of the coat protein N-terminal segment in *Potato virus X* virion stability and functional activity

ELENA LUKASHINA¹, ALEXANDER KSENOFONTOV¹, NATALIA FEDOROVA¹, GENNADY BADUN², ANNA MUKHAMEDZHANOVA³, OLGA KARPOVA³, NINA RODIONOVA^{3,†}, LYUDMILA BARATOVA¹ AND EVGENY DOBROV^{1,*}

¹A.N. Belozersky Institute of Physico-Chemical Biology, Moscow State University, 119992 Moscow, Russia

²Department of Chemistry, Moscow State University, 119992 Moscow, Russia

³Department of Biology, Moscow State University, 119992 Moscow, Russia

SUMMARY

Previously, we have reported that intact *Potato virus X* (PVX) virions cannot be translated in cell-free systems, but acquire this capacity by the binding of PVX-specific triple gene block protein 1 (TGBp1) or after phosphorylation of the exposed N-terminal segment of intravirus coat protein (CP) by protein kinases. With the help of *in vitro* mutagenesis, a nonphosphorylatable PVX mutant (denoted ST PVX) was prepared in which all 12 S and T residues in the 20-residue-long N-terminal CP segment were substituted by A or G. Contrary to expectations, ST PVX was infectious, produced normal progeny and was translated *in vitro* in the absence of any additional factors. We suggest that the N-terminal PVX CP segment somehow participates in virion assembly *in vivo* and that CP subunits in ST virions may differ in structure from those in the wild-type (UK3 strain). In the present work, to test this suggestion, we performed a comparative tritium planigraphy study of CP structure in UK3 and ST virions. It was found that the profile of tritium incorporation into ST mutant virions in some CP segments differed from that of normal UK3 virions and from UK3 complexed with the PVX movement protein TGBp1. It is proposed that amino acid substitutions in ST CP and the TGBp1-driven remodelling of UK3 virions induce structural alterations in intravirus CPs. These alterations affect the predicted RNA recognition motif of PVX CP, but in different ways: for ST PVX, labelling is increased in α -helices 6 and 7, whereas, in remodelled UK3, labelling is increased in the β -sheet strands β 3, β 4 and β 5.

INTRODUCTION

In 2001, it was found in our laboratory that intact particles of flexuous helical *Potato virus X* (PVX) are incapable of *in vitro* translation in different cell-free extracts, but acquire the capacity to be translated by a *Tobacco mosaic virus* (TMV)-like, cotranslational,

disassembly mechanism after phosphorylation of the N-terminal segment of the coat protein (CP) in the virions by plant T–S protein kinases (PKs). After PK treatment, this surface-exposed segment imparts to the PVX virions the ability to bind ribosomes to the particle end containing the intravirus RNA 5′-terminus. Following this binding, CP subunits somehow change their structure, and ribosomes begin translation of the 5′-terminal PVX replicase gene, releasing CP subunits step by step during translation (Atabekov *et al.*, 2001, 2007; Rodionova *et al.*, 2003). During the course of this research, a PVX mutant was constructed in which all 12 S and T residues of the 20-residue-long N-terminal sequence of the CP were substituted by A or G. This mutant (denoted ST PVX) could not be phosphorylated *in vitro* by protein kinase C (PKC), but produced infectious progeny. It was found that, in contrast with UK3, ST virions were translated in a wheat germ extract cell-free system with an efficiency similar to that of an equal amount of free PVX RNA. This translational activity was not affected by trypsin removal of the 19 N-terminal ST CP residues, whereas the untreated UK3 virions were not translated either before or after trypsin treatment (Atabekov *et al.*, 2001, 2007; Kozlovsky *et al.*, 2003; Rodionova *et al.*, 2003). It should be stressed that, after removal, the N-terminal segment of ST and UK3 virions still retained differences in translational capacity, although, after trypsinolysis, their CP should have an almost identical primary structure, excluding T–A substitution at the first position. Potexvirus CP N-terminal tail sequences are highly divergent, and some contain alanine in corresponding positions (Lan *et al.*, 2010). This should mean that the N-terminal segment of UK3 CP is involved in virion assembly *in vivo*, and the virions assembled with or without normal CP N-terminal segment participation are structurally different.

This suggestion received some support in experiments on *in vitro* translation with or without the oligonucleotide complementary to the PVX RNA 5′-terminal nucleotides 22–36. This oligonucleotide completely prevented the translation of isolated PVX RNA, ST virions and the phosphorylation-activated normal PVX, but did not inhibit the PVX virion translation induced by remodelling with the PVX nonvirion movement protein triple gene block protein 1 (TGBp1) (Atabekov *et al.*, 2001, 2007; Kozlovsky *et al.*, 2003; Rodionova *et al.*, 2003). These data demonstrate that ST

*Correspondence: Email: dobrov@belozersky.msu.ru

†Deceased on 14 April 2011.

virions resemble, in their translational activity, the phosphorylated wild-type PVX virions, but not the virions remodelled by binding of TGBp1. The ST virions and their CP also displayed other unusual properties, testifying to multiple roles of the PVX CP N-terminal sequence in virus assembly and functions (Karpova *et al.*, 2006).

In this work, we used a tritium planigraphy technique to elucidate the structural differences between the ST and wild-type PVX virions. We have employed tritium planigraphy previously in studies of several helical plant viruses, their mutants and complexes with proteins for which the use of direct methods (X-ray diffraction and nuclear magnetic resonance) was impossible (see, for instance, Baratova *et al.*, 2001; Dobrov *et al.*, 2003, 2004, and Goldanskii *et al.*, 1988, where the main principles, advantages and disadvantages of tritium planigraphy can be found).

In tritium planigraphy, samples (proteins, virus particles or nucleic acid–protein complexes) are bombarded at -196°C by atomic tritium heated to 2000 K, thoroughly purified of nonspecific label and hydrolysed to peptides by trypsin (and/or other proteases). The peptides obtained are separated by high-performance liquid chromatography (HPLC), hydrolysed by HCl, and their amino acid content and tritium radioactivity are measured. These data enable us to estimate the radioactivity of each amino acid in the protein. Based on radioactivity levels for different segments of the polypeptide chain, the possible protein tertiary structure can be predicted, assuming that segments of higher radioactivity are located closer to the protein (virion, complex) surface. Recently, we published the results of a comparative tritium planigraphy study of untreated PVX virions and PVX virion complexes with the PVX-specific movement protein TGBp1 (Lukashina *et al.*, 2009).

RESULTS

Figure 1 presents the sequence of 25 N-terminal amino acids of UK3 PVX and ST PVX CPs with all 12 S and T residues in the mutant CP substituted by A or G (Kozlovsky *et al.*, 2003). It should be remembered that, as shown by Baratova *et al.* (2004), the N-terminal serine of UK3 CP is glycosylated and UK3 virions possess a strong water column on their surface, whereas ST virions do not contain carbohydrate residues in their CP and have a much less strongly ordered water column.

To elucidate the possible structural mechanisms of functional changes observed in experiments on ST virion translation (Atabekov *et al.*, 2007; Kozlovsky *et al.*, 2003), we employed tritium planigraphy. Recently, we have reported the results of a similar

UK3: SAPASTTQPI GSTTSTTTKT AGATP–

ST: AAPAGGAQPI GAGGAAGAKA AGATP–

Fig. 1 N-terminal amino acid sequences of the coat proteins of UK3 *Potato virus X* (PVX) and ST mutant; amino acid substitutions are underlined (Kozlovsky *et al.*, 2003).

study in which tritium incorporation was compared for the untreated UK3 virions and their complexes with PVX movement protein TGBp1 (Lukashina *et al.*, 2009).

The ST mutant samples were irradiated in the same experiments, in parallel with the UK3 + TGBp1 complexes. In each of the three experiments performed, three samples (UK3, UK3 + TGBp1 and ST) were irradiated in parallel and under conditions (irradiation dose and intensity, temperature, sample concentration, etc) that were as closely similar as possible. HPLC tryptic peptide separation represents the least reproducible part of tritium planigraphy, and the best of the three experiments (see below) is considered to be that in which the best separation of peptides was observed in the three samples (UK3, ST and UK3 + TGBp1).

Recently, a new system has been developed that allows the parallel on-line determination of any amino acid content (from ninhydrin staining data) and tritium radioactivity in any hydrolysed peptide (Lukashina *et al.*, 2009). The use of this technique enabled significant improvement in measurement accuracy (compare, for instance, our data in Baratova *et al.*, 2001 and Lukashina *et al.*, 2009).

Figure 2 demonstrates tritium label distribution along the polypeptide chain for the 236-residue-long wild-type PVX (UK3) and ST mutant CPs after tritium bombardment of the intact virions. The results of the best of the three experiments are shown. Data for the wild-type virus are those presented in our previous publication (Lukashina *et al.*, 2009). The predicted trypsin cleavage sites are shown under the amino acid sequence of the UK3 CP (N-terminal sequences of the UK3 PVX and ST mutant CPs shown in Fig. 1). First, as can be seen in Fig. 2, we successfully separated and identified all ST mutant and wild-type CP tryptic peptides, with the exception of di- and tri-peptides (T8, T16, T18, and T5, T15). We also did not include the data for cysteine and tryptophan residues, which were degraded on acid hydrolysis.

The experimental data are the average values of tritium incorporation for each type of amino acid residue in each tryptic peptide. Thus, for example, peptide T3 (A46–K60) contains four alanine residues (positions 45, 48, 51, 53), and their molar radioactivity values are considered to be equal, each constituting one-quarter of the total value of the alanine radioactivity in peptide T3. Therefore, the contribution of an individual residue cannot be determined unless it is the only one of its type in the peptide; this problem has been discussed in our previous publications (see, for instance, Dobrov *et al.*, 2004 and Lukashina *et al.*, 2009). In addition, in tritium planigraphy, we do not compare individual residues, but only relatively long protein segments, because we search for entire regions with the most pronounced general tendency to accessibility change. For this reason, the data from Fig. 2 are summarized in Table 1, the analysis of which allowed us to draw further conclusions on structural alterations in ST CP.

Figure 3 shows the latest variant of our hypothetical three-dimensional model of the PVX CP structure *in situ* in its 'sandwich'

Table 1 Tritium label distribution among predicted coat protein (CP) secondary structure elements in UK3 and ST. The summed element radioactivity was calculated by summing the radioactivity of all amino acid residues comprising a predicted element. The averaged element radioactivity was calculated by dividing the summed element radioactivity by the number of residues comprising an element, i.e. the average radioactivity of one residue in an element was evaluated.

1	2	3	4	5	6	7	8
Secondary structure element	Amino acid residues	Number of residues	Summed element radioactivity (%)		Averaged element radioactivity (%) per residue		Relative radioactivity change in ST
			ST	UK3	ST	UK3	
β 1	1–8	8	3.09	5.39	0.39	0.67	0.57
/1	9–14	6	2.60	3.73	0.43	0.62	0.70
β 2	15–22	8	1.68	1.16	0.21	0.14	1.45
/2	23–24	2	0.17	0.10	0.08	0.05	1.75
α 1	25–34	10	1.48	0.95	0.15	0.10	1.55
/3	35–39	5	0.91	0.48	0.18	0.10	1.89
α 2	40–51	12	2.44	2.05	0.20	0.17	1.19
/4	52–54	3	1.85	2.02	0.62	0.67	0.92
α 3	55–70	16	7.10	7.32	0.44	0.46	0.97
/5	71	1	1.39	1.31	1.39	1.31	1.06
α 4	72–102	31	27.56	31.17	0.89	1.01	0.88
/6	103–108	6	2.22	2.26	0.37	0.38	0.98
α 5	109–135	27	10.18	12.12	0.38	0.45	0.84
/7	136–144	9	0.61	0.41	0.07	0.05	1.51
β 3	145–152	8	0.50	0.32	0.06	0.04	1.56
/8	153–156	4	0.18	0.14	0.04	0.03	1.27
α 6	157–175	19	4.36	2.24	0.23	0.12	1.95
/9	176–184	9	5.50	5.43	0.61	0.60	1.01
β 4	185–191	7	1.64	1.76	0.24	0.25	0.94
/10	192–194	3	2.03	2.42	0.68	0.81	0.84
β 5	195–202	8	1.64	1.68	0.21	0.21	0.98
/11	203–205	3	0.64	0.47	0.21	0.16	1.36
α 7	206–220	15	7.08	4.50	0.47	0.30	1.57
/12	221–227	7	7.92	5.96	1.13	0.85	1.33
β 6	228–234	7	4.24	3.84	0.61	0.55	1.11
C	235–236	2	1.00	0.76	0.50	0.38	1.30

variant [with the proposed $\beta\alpha\beta\beta\alpha$ RNA-recognition motif (RRM) (Auweter *et al.*, 2006) domain located above the α -helical bundle (Lukashina *et al.*, 2009; Nemykh *et al.*, 2008)]. The positions of all the predicted secondary structure elements—the α -helices (h), the β -strands (s) and loops (l)—are presented in Fig. 2 and columns 1–3 of Table 1. Figure 2 and Table 1 demonstrate that, for some parts of the CP sequence (for instance, along the 40–156-residue region), very good agreement (up to a few percentage points) was observed between the ST and wild-type PVX data. Undoubtedly, such accuracy was achieved owing to the use of the parallel determination of ninhydrin staining and tritium radioactivity.

However, for some segments of the CP sequence, significant differences in labelling between ST and UK3 were observed, and these segments did not coincide with those demonstrating different tritium incorporation in the case of the UK3/UK3 + TGBp1 pair (Lukashina *et al.*, 2009).

First, a pronounced drop in radioactivity in the ST CP was observed for the N-terminal β 1-strand (residues 1–8) and loop /1 (9–14). This region displayed a high label content in both CPs making the observed difference highly reliable (Fig. 2). This is seen more clearly in Table 1 (columns 4–8), where the label content has been calculated per amino acid residue in the secondary structure elements predicted by the ‘sandwich’ variant of our model of the

Fig. 2 Tritium label distribution in ST and UK3 coat protein (CP) polypeptide chains obtained on atomic tritium bombardment. Experimental data are shown as A_i/A_{Σ} , the ratio of the given amino acid residue molar radioactivity (A_i) to the sum of the radioactivity of all residues in the protein (A_{Σ}), and are expressed as a percentage. Data on UK3 are represented as white columns and data on ST as grey columns. The UK3 CP amino acid sequence is given as a single-letter code; the numbers of the amino acid residues from the N-terminus are shown in tens. The trypsin cleavage sites are indicated by arrows; the numbers of tryptic peptides are presented with numbering corresponding to all possible predicted peptides. Molar radioactivities are the average values of tritium label incorporation for each type of amino acid residue in each tryptic peptide. The positions of predicted secondary structure elements are shown below the sequence: α -helices are represented as white rectangles, β -strands as grey arrows and unstructured loops as black lines. The 236-residue-long sequence of the *Potato virus X* (PVX) CP is split into three parts: 1–80 amino acid residues (A), 81–160 amino acid residues (B) and 161–236 amino acid residues (C).

Fig. 3 'Sandwich' variant of the spatial structural model of the *Potato virus X* coat protein (PVX CP) subunit in a virion. The RNA-recognition motif (RRM) consists of $\beta 3$, $\alpha 6$, $\beta 4$, $\beta 5$ and $\alpha 7$. The virion long axis is located on the left and the RRM β -sheet at the top. The α -helices are shown as cylinders and the β -strands as arrows, apart from probable $\beta 2$. The N- and C-termini of the protein chain are indicated. Domain swapping may occur through hinges I/3 (between $\alpha 1$ and $\alpha 2$) and I/7 (between $\alpha 5$ and $\beta 3$). These hinges are highlighted in light grey. This model was published in Lukashina *et al.* (2009).

intravirus PVX CP structure (Fig. 3). In $\beta 1$, the ratio of tritium incorporation for ST CP constitutes only 0.57 of that for UK3 $\beta 1$ and, for I/1, this ratio is 0.70 (Table 1, column 8). This change may be a result of partial burial of the previously exposed CP N-terminal segment, caused by the substitution of highly polar S and T residues by less polar G and A, and/or by the absence of carbohydrate residues in the ST CP (Baratova *et al.*, 2004).

A significant increase in the label content in ST CP relative to UK3 CP was also found in subsequent regions of the molecule: $\beta 2$, I/2, $\alpha 1$ and I/3 (see Fig. 2 and Table 1). Of particular interest was the almost twofold increase in the ST/UK3 labelling ratio for loop I/3, which corresponds to one of the two hinge regions causing the proposed domain swapping in the PVX intravirus CP (Lukashina *et al.*, 2009; Nemykh *et al.*, 2008). The ST/UK3 ratio for I/3 was very high, but it should be remembered that absolute values of radioactivity in this region were low (Table 1, columns 6 and 7). The relative increase in tritium content for $\beta 2$ and $\alpha 1$ was smaller than for I/3 (column 8), but radioactivities themselves were much higher (columns 6 and 7). In the case of the TGBp1–UK3 complex, we observed some decrease in tritium accessibility for the 1–24 CP region (Lukashina *et al.*, 2009); however, this decrease was not as pronounced and did not change to an increase as observed in the present work for the $\beta 2$ to I/3 segments.

As mentioned above, for the $\alpha 2$ to I/8 region (residues 40–156), no difference was observed in tritium content between the ST and UK3 CPs. However, the greatest increase in labelling (ratio of 1.95) was observed for the $\alpha 6$ -helix or, more specifically, its C-terminal

half (residues 166–175), whereas, for the 157–165 fragment, very low labelling was observed for both proteins. One further region of similar radioactivity (residues 176–205) was found. This was followed by the $\alpha 7$ -helix region (206–220), which contains a larger amount of label in the case of ST. Absolute tritium levels in this region were relatively large. The $\alpha 6$ - and $\alpha 7$ -helices are separated in the PVX CP sequence but, according to our model, both should belong to RRM (Fig. 3).

The increase in tritium incorporation in the 176–198 CP segment, characteristic of the PVX–TGBp1 complex (Lukashina *et al.*, 2009), was not observed in the case of the ST mutant (Fig. 2). In addition, no difference between ST and the wild-type PVX was found in the label incorporation in the 136–156 region characterized by low tritium content. This supports the reliability of the large difference in labelling observed in this region for the PVX–TGBp1 complex (Lukashina *et al.*, 2009).

The data in Table 1 are grouped in accordance with our hypothetical model of the three-dimensional structure of the intravirus PVX CP. However, the values in Fig. 2 are not based on any predictions and should remain a measure of tritium accessibility of the PVX CP residues, independent of any models.

In the case of the ST mutant, as with the wild-type PVX and the PVX–TGBp1 complex, we found no individual T7 and T11 peptides, because of their probable cross-linking by a C86–C126 disulphide bond. However, as discussed previously (Lukashina *et al.*, 2009), we cannot be certain that this bond is present in native PVX particles. In addition, it should be remembered that the tritium planigraphy data are, in principle, averaged over the total length of the virion and cannot be used to distinguish situations in one or several subunit turns at the particle ends from the total number of about 130 turns.

DISCUSSION

In 2000, we described two different mechanisms of PVX virion translational activation observed in our laboratory. The first mechanism was cotranslational disassembly after phosphorylation of the PVX CP N-terminal segment, as described in the Introduction, and the second mechanism was called 'remodelling' and took place after binding of the PVX-encoded nonvirion movement protein TGBp1 to the helical particle end, containing the 5'-terminus of intravirus RNA. On remodelling, after TGBp1 binding, instantaneous destabilization of the entire 515-nm-long PVX particle occurs, thus reflecting gross changes in the intravirus CP structure, which, however, do not in themselves result in virion disruption (Atabekov *et al.*, 2000, 2007; Rodionova *et al.*, 2003; Torrance *et al.*, 2009). It was assumed that both mechanisms are used during the course of PVX infection, but at different stages (Atabekov *et al.*, 2007). The results of our tritium planigraphy study of TGBp1 interaction with PVX virions (remodelling) can be found in Lukashina *et al.* (2009), and the results of our tritium

planigraphy comparative study of UK3 and ST PVX are presented in this article.

As has been shown in the study of Zayakina *et al.* (2008), deletion by *in vitro* mutagenesis of only 10 C-terminal residues of the PVX CP results in the loss of virion ability to bind TGBp1 to its 5'-terminal turn. This suggests that the CP subunits in the PVX virions are packed in such an orientation that their C-terminal sequences face the outer surface of the virion 5'-end. This means that, if our model of PVX CP structure *in situ* (Fig. 3) is correct, the RRM domain should be located on this surface.

We assumed that the PVX CP N-terminal segment plays the role of a scaffold during the PVX virion assembly in *Datura stramonium* and, in the absence of this segment, virions with certain types of defect are formed. A similar conclusion was drawn by Leclerc's group for the potexvirus *Papaya mosaic virus* (Laliberté Gagné *et al.*, 2008), and by Anindya and Savithri (2003) on the basis of their study of the assembly of the potyvirus *Pepper vein banding virus*.

In this case, the proposed N-terminal phosphorylation-induced or amino acid substitution-induced alterations in the 5'-surface-located layer of the CP subunits may increase the accessibility of the subunits to some of the many known (and, as yet, unknown) participants in the initiation of the cotranslational disassembly process.

The tritium planigraphy data (excluding the very different mutated N-terminal region) show that label incorporation reliably increases only in the $\alpha 6$ - and $\alpha 7$ -helices that form the α -helical layer of the RRM (Fig. 3). In the PVX-TGBp1 complex, tritium incorporation in these helices does not change, but strongly increases in almost all segments forming the RRM β -sheet (Lukashina *et al.*, 2009). This suggests that the RRM position is altered in both of these cases, but in different ways. In other regions of the intravirus ST CP, no changes in labelling are seen. To demonstrate this more clearly, Fig. 4 shows tritium incorporation (per residue) for all elements of our model in the untreated UK3 PVX, ST PVX and UK3 PVX complex with TGBp1 (Lukashina *et al.*, 2009).

The findings made by Atabekov's group (Atabekov *et al.*, 2000, 2001, 2007; Kozlovsky *et al.*, 2003; Rodionova *et al.*, 2003) suggest the existence of three functional states of PVX virions: (i) native (untranslatable *in vitro*) virions; (ii) virions remodelled by TGBp1 binding (quickly and completely disrupted by different agents or after translation of several 5'-terminal codons of PVX RNA); and (iii) PVX virions made translatable by phosphorylation of the CP N-terminal segment or numerous amino acid substitutions in the ST mutant, and relatively slowly disrupted by the TMV-like cotranslational disassembly mechanism.

We recently hypothesized (Lukashina *et al.*, 2009; Nemykh *et al.*, 2008) that the intravirus PVX CP may undergo domain swapping, and this swapping may occur through hinges $\beta 3$ and $\beta 7$ connecting the α -helix bundle and RRM (Fig. 3). The results of the

Fig. 4 Tritium label distribution in the predicted coat protein (CP) secondary structure elements for ST (white), untreated UK3 (grey) and the UK3-TGBp1 complex (black). The data for the *Potato virus X* (PVX) triple gene block protein 1 (TGBp1) complex are from Lukashina *et al.* (2009). For the predicted element position, see Table 1. The element tritium content is divided by the element length (i.e. the data are shown per amino acid residue).

present and previous work (Lukashina *et al.*, 2009) suggest that RRM changes its location and/or structure in both PVX virion translatable states, but in different ways.

The most straightforward interpretation suggests that the domain-swapped structure should correspond to the most stable (and, supposedly, the most tightly packed) native PVX virions, and that, in both remodelled and phosphorylated virions, this swapping disappears. In this case, in more loosely packed TGBp1-remodelled particles, tritium accessibility of the RRM β -sheet (Fig. 3), supposedly located on the intersubunit axial interface, increases (see Lukashina *et al.*, 2009). Phosphorylation (or amino acid substitutions in ST) should induce not only a looser packing, but also RRM rotation around its long axis, resulting in stronger labelling of the RRM $\alpha 6$ - and $\alpha 7$ -helices (present work).

The 'sandwich' variant of our intravirus PVX CP model, as depicted in Fig. 3, with the RRM domain located on the upper surface, and the N- and C-termini on the outer side of the virion, and the hinges located in the interior of the particle, appears to be particularly suitable for domain swapping. This swapping should take place during virion assembly (see above) and occur between laterally adjacent subunits from one CP molecule to the next, in a 'domino effect.' On TGBp1 binding to the virion 5'-end (or on N-terminal segment phosphorylation), a fast wave of cooperative breaking of intersubunit interactions in domain-swapped 'dimers' should occur with their replacement by 'normal' intersubunit interactions, with a concomitant decrease in virion stability. In phosphorylation-induced translational activation, this process should be accompanied by RRM domain rotation, relocating $\alpha 6$ - and $\alpha 7$ -helices on the subunit surface.

It seems that the PVX CP N-terminal region is highly important in virus infection (Atabekov *et al.*, 2001, 2007; Kozlovsky *et al.*, 2003; Rodionova *et al.*, 2003), and probably belongs to the class of natively unfolded segments (Goodman, 1975; Nemykh *et al.*, 2007 and references cited therein). Nevertheless, despite its 'unfoldedness' and surface location, it often displays high resistance to proteolytic digestion. Some purified PVX preparations (those that contain only the undegraded Ps form of the protein) lose the CP N-terminal segment rather slowly at +4 °C and completely preserve the Ps form after years of storage at –20 °C in 50% glycerol (Goldstein *et al.*, 1990). The reasons for this stability remain unknown, but most probable is the suggestion that the carbohydrate moiety at the PVX intravirus CP N-terminus is much larger than observed in the study by Baratova *et al.* (2004), but most of this moiety is lost during the course of handling and, especially, during mass spectrometry.

Following from this work and previous results (Atabekov *et al.*, 2000, 2001, 2007; Rodionova *et al.*, 2003), the PVX virions exist in three different functional and structural states. In addition, the PVX virions may undergo one more structural transition on incubation at –20 °C in low ionic strength solutions (Nemykh *et al.*, 2008), testifying to the surprisingly high plasticity of the PVX virion structure.

EXPERIMENTAL PROCEDURES

Preparation of ST PVX mutant with substitutions of serine and threonine in CP

The mutant was constructed on the basis of plasmid pPVX-GFP containing a full-length cDNA copy of the strain UK3 PVX under the control of the 35S promoter (Fedorkin *et al.*, 2000). The PVX genomic fragment inserted between sites *Bsp*1 and *Spe*1 was subcloned into plasmid pGEM5Zf(–). This construct was used for polymerase chain reaction (PCR) with the standard primer 5'-d(GTAAACGACGGCCAGT)-3' and primer M-ST:5'-d(GCGCTGCAGCTTTGCGCCAGCTGCGCCACCTGCCCAATTGGCTGTGC TCCGCCAGCTGGTGCTGCCATCTTCGAGT)-3'. The mutant full-length cDNA copy of PVX with the substitution of the initial N-terminal domain of the PVX CP sequence MSAPASTTQPIGSTTTTKTAG by sequence MAAPAGGAQPIGAGGAAGAKAAG was prepared on the basis of the PCR fragment. The resulting construction (referred to as ST PVX) was used to infect *Nicotiana benthamiana* plants (Kozlovsky *et al.*, 2003). The ST mutant remained stable after four passages in *N. benthamiana*.

Virus preparations

UK3 and ST PVX were isolated from leaves of *D. stramonium* as described by Atabekov *et al.* (2000). The yield of ST PVX reached 0.3–0.5 g/kg of leaves and was similar to that obtained for wild-type PVX.

Tritium planigraphy

Briefly, the UK3 and ST samples (0.65 mL, 2 mg/mL) in a special device were irradiated by three 10-s pulses of hot tritium atoms obtained from

molecular tritium gas (0.5 Pa) on a tungsten filament heated to 2000 K. To this end, the samples were evenly distributed on the inner surface of glass vessels and cooled with liquid nitrogen. Before each tritium exposure, a vacuum was applied. After labelling, the virus samples were lyophilized, resuspended in buffer solution and centrifuged at 20 000 *g* for possible degradation product removal. Next, PVX CP was isolated using a standard LiCl method (Goodman, 1975), dialysed three times against 2–3 L of deionized water and stored at 4 °C in 10 mM Tris/HCl, pH 7.5. At this stage, exchangeable label (in O-³H, N-³H and S-³H bonds) was removed without the loss of label in the C-³H bonds, in which we were interested.

Directly after labelling, the amount of tritium label in the samples was approximately 10¹³ cpm/mL. This was reduced to about 10⁶ during amino acid analysis (see below).

UK3 and ST CP samples were then treated with Promega (Madison, WI, USA) sequencing-grade trypsin, diluted with starting eluent (0.1% trifluoroacetic acid in water) and the peptides were separated by reverse phase (RP)-HPLC using a 0%–60% acetonitrile gradient in water (in 73 min). The separated PVX and ST CP tryptic peptides were subjected to acid hydrolysis (Tsugita and Scheffler, 1982), and amino acid analysis was performed on a Hitachi (Tokyo, Japan) L-8800 analyser with ninhydrin derivatization. A Packard Padiomatic 150 TR (Hewlett-Packard, Palo-Alto, CA, USA) flow scintillation analyser was used for the simultaneous measurement of amino acid content and levels of tritium radioactivity. The liquid scintillation cocktail Ultima-Flo AP (flow rate, 0.75 mL/min) was mixed with the derivatized column eluate, and scintillations were counted in a 0.35-mL flow cell. The radioactivity counts and optical density at 570 and 440 nm were processed together.

The molar radioactivities for each type of amino acid residue in a peptide were calculated by dividing the amino acid radioactivity by its molar quantity. The values of tritium incorporation for each type of amino acid residue in each tryptic peptide were normalized using special coefficients reflecting the probability of label incorporation according to the nature of the residue (Gedrovich and Badun, 1992).

A more detailed description of tritium planigraphy technology can be found in Lukashina *et al.* (2009).

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