Virion glycosylation governs integrity and infectivity of infectious pancreatic necrosis virus

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Abstract

The possible importance of the *O*-linked glycosylation in virion stability and infectivity of infectious pancreatic necrosis virus (IPNV) was analysed. Enzymatic treatment with *O*-glycosidase of radio-labelled virions under different ionic conditions, to allow for possible alternative exposure of glycosidic enzyme cleavage sites, did not alter the specific infectivity of virions re-isolated after rate-zonal centrifugation in glycerol gradients. As an alternative method to assess the significance of carbohydrates in IPNV integrity, periodate oxidation in the presence of an aldehyde quencher was chosen. Following re-isolation of viruses, a 3–5 ¹⁰log-unit reduction in specific infectivity was revealed and, at higher concentrations, a total disruption or virion aggregation was observed. The loss of infectivity of intact virions was not because of a lack of attachment to cells. Additionally, re-evaluation of reading values from UV-spectra of purified IPNV yielded a specific infectivity of 3×10^{11} TCID₅₀-units mg⁻¹ of protein and a ratio of 40 virions per TCID₅₀-unit in the CHSE-214 cell system.

Keywords: glycosylation, infectious pancreatic necrosis virus, infectivity, integrity, periodate treatment.

Introduction

Infectious pancreatic necrosis virus (IPNV), the type species of the genus *Aquabirnavirus* within the

family *Birnaviridae*, is composed of a single-shelled capsid enclosing two linear segments of double-stranded RNA (Dobos 1995). The larger segment A encodes a polyprotein containing a protease, VP4, that auto-catalytically liberates the two structural proteins, the precursor of the coat protein VP2 (Duncan, Nagy, Krell & Dobos 1987) and the core protein VP3 (Hjalmarsson, Carlemalm & Everitt 1999). A second overlapping open reading frame encodes a protein denoted VP5, the role of which is not yet fully understood (Hong, Gong & Wu 2002; Santi, Sandtrø, Sindre, Song, Hong, Thu, Wu, Vakharia & Evensen 2005b; Santi, Song, Vakharia & Evensen 2005a). The viral polymerase, and likewise the genome-linked primer, is encoded by segment B (Dobos 1995). IPNV is a serious pathogen and threat to the commercial aquaculture industry and continues to spread into new fish farms causing substantial economic losses (Rodriguez Saint-Jean, Borrego & Perez-Prieto 2003). When first isolated, IPNV was considered to be a reovirus based on the nature of the viral genome (Moss & Gravell 1969), but further insights into virion structure placed it within a new taxonomic family (Dobos, Hill, Hallett, Kells, Becht & Teninges 1979) that was approved by the ICTV in 1986 (Brown 1986). When quantifying the amount of purified IPNV in solution by UV readings, the relationship originally determined for reoviruses is still employed, despite differences in protein and nucleic acid composition (Smith, Zweerink & Joklik 1969; Dobos, Hallett, Kells, Sorensen & Rowe 1977; Dobos *et al.* 1979).

In this laboratory, we have observed an unpredictable destabilization of IPNV after short-term storage in the cold that might mirror the inherent

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ability of virions to easily disassociate. This ability could be a reflection of the concept of metastability, referring to the property of viral capsids to remain intact and protect the viral genome during the extracellular phase until entry into a susceptible cell where subsequent uncoating is pivotal for virus replication. Documented factors influencing virion integrity are fluctuations in temperature, ionic concentration and pH (Witz & Brown 2001; Johnson 2003). IPNV entry occurs via receptor-mediated endocytosis (Couve, Kiss & Kuznar 1992; Kuznar, Soler, Farias & Espinoza 1995; Granzow, Weiland, Fichtner & Enzmann 1997), but unlike many other viruses, it does not require an acidic pH to exit the endosome (Espinoza & Kuznar 1997).

The majority of glycosylated proteins carry *N*-linked glycosylation, a post-translational modification that occurs in the endoplasmic reticulum. Less abundant are structural viral proteins with an *O*-linked modification. However, the adenovirus fibre, responsible for cellular recognition and attachment (Philipson, Lonberg-Holm & Pettersson 1968; Ishibashi & Maizel 1974) and the coat protein of IPNV, VP2 are both glycosylated in an *O*-linked fashion (Hjalmarsson *et al.* 1999). The significance of this post-translational modification of the replicative cycle of IPNV is not known. The M protein of the envelope of the coronavirus mouse hepatitis virus is invariably *O*-glycosylated, and it is not important for virulence or replication (de Haan, de Wit, Kuo, Montalto, Masters, Weiss & Rottier 2002). The multifaceted large T-antigen of the polyomavirus SV40, essential for viral gene regulation and expression, is *O*-glycosylated in the region important for the nuclear localization signal, retinoblastoma recognition and viral replication (Medina, Grove & Haltiwanger 1998; Ahuja, Saenz-Robles & Pipas 2005). *O*-glycosylated nucleocytoplasmic proteins have also been implemented in signal transduction events and as such would influence protein–protein interactions (Wells, Vosseller & Hart 2001). This raises an interesting question on the possible functional interplay between mutual phosphorylation and glycosylation sites. In view of the broad spectrum of the events dependent on *O*-linked glycosylation, it would be of interest to investigate whether the *O*-linked glycosylation in IPNV has any impact on, e.g. virion stability and viral infectivity.

In the present investigation, we show that virions of IPNV upon oxidative treatment with sodium periodate either disaggregate or display a dramatic

loss of infectivity of re-isolated viruses. Attempts by enzymatic treatment with *O*-glycosidase to remove the presence of carbohydrates were not successful. We also present an alternative relationship between UV-absorbance data and protein content of purified IPNV preparations where a ratio of 40:1 of virions over TCID₅₀ infectious units is obtained.

Materials and methods

Cell maintenance and propagation

Chinook salmon embryo cells (CHSE-214) were grown as monolayer cultures at 16–18 °C in tightly sealed 175-cm² cell culture flasks (Sarstedt Inc.) in 20 mL of E-MEM (Gibco, Invitrogen Corp.) supplemented with 2 mM L-glutamine, 0.1 mM non-essential amino acids (NEAA), 10 mM HEPES (4-(2-hydroxyethyl) piperazineethanesulphonic acid) (pH 7.0), 1 mg sodium bicarbonate mL⁻¹, 20 µg of gentamicin mL⁻¹ (Gibco) and 10% foetal bovine serum (FBS; Hyclone).

Propagation of viruses

Propagation of IPNV, serotype West Buxton VR-299 (Espinoza, Farias, Soler & Kuznar 1985; Hill & Way 1995) was performed in E-MEM supplemented with 1% FBS. For infection, a single IPNV seed pool was used to avoid the emergence of viral quasi-species (Santi, Vakharia & Evensen 2004). Cells were infected at a multiplicity of infection (MOI) of 3 TCID₅₀/cell, and the infection was allowed to manifest itself for 3 days when a fully developed CPE was evident. Viral isolation and purification was carried out by PEG 6000 precipitation and further as described by Dobos *et al.* (1979). In short, precipitated virions were dissolved in TNE (100 mM NaCl, 1 mM EDTA in 10 mM Tris–HCl, pH 7.5) and subjected to rate-zonal centrifugation at 77 100 *g*_{av} for 2 h at 10 °C in an SW 41 rotor in discontinuous gradients composed of a high-density CsCl cushion overlaid by CsCl (1.30 g mL⁻¹ in 50 mM Tris–HCl, pH 8.1) and 20% (w/v) sucrose in TNE-buffer. Recovered virus was subjected to isodensity ultracentrifugation at 49 300 *g*_{av} overnight at 10 °C in an SW 41 rotor in linear CsCl gradients, ranging from 1.25–1.40 g mL⁻¹ of CsCl in 50 mM Tris–HCl, pH 8.1. Purified viruses were stored at 4 °C in a stabilizing buffer (SB; 150 mM NaCl, 1 mM MgCl₂, 5% glycerol in 10 mM Tris–HCl, pH 7.5).

Radiolabelling of viruses

Cell monolayers of 80% confluence were infected at a MOI of 5 in gentamicin- and NEAA-free medium. Two hours post-infection cells (p.i.) were washed, and ^3H -mannose or a ^3H -amino acid mixture and ^{32}P -orthophosphate was added to 15 mL of fresh growth medium per 175-cm² flask yielding 33 μCi of ^3H -mannose mL⁻¹, 10 μCi of ^3H -amino acids mL⁻¹ and 13 μCi of ^{32}P mL⁻¹ of medium. Viruses were harvested 65 h p.i. and further purified as described earlier. The mean specific activities of purified viral pools were 120, 3100–4700 and 1840 cpm μg^{-1} IPNV protein for ^3H -mannose, ^3H -amino acids and ^{32}P -orthophosphate, respectively. Viral pools were also radiolabelled separately with only ^3H -amino acids.

Viral infectivity titration

CHSE-214 cells were seeded in 96-well cell culture plates (Costar, Corning) to yield 10⁴ cells per well. Cells were kept in a cell culture chamber where CO₂ levels and pH could be controlled by intermittent addition of solid citric acid and sodium bicarbonate at a ratio of 1:2.25 in water. Two days post-seeding and at a cell density of approximately 80%, the cells were challenged with IPNV diluted serially tenfold in cell culture medium supplemented with 1% FBS. Each row of eight wells was administered 200 μL per well from an assigned viral dilution. Following 3–5 days of incubation, the cell culturing medium was carefully aspirated and the cells were fixed with 1% formaldehyde in phosphate-buffered saline (PBS; 0.14 M NaCl, 3 mM KCl in 0.01 M phosphate buffer, pH 7.4) for 30 min and subsequently stained with 0.05% crystal violet in 50% methanol for 30 min. Excess dye was removed, and the plates were rinsed gently under running tap water. In wells positive for viral infection, all cells were lysed and the remains became unstained. The viral titre, expressed as TCID₅₀ mL⁻¹, was determined according to Reed & Muench (1938).

Viral attachment assay

CHSE-214 cells were grown to confluency in 6-well plates (Sarstedt Inc.). After washing once in Hank's balanced salt solution, HBSS (Gibco), the same amounts of radiolabelled virus (160 ng of total virion protein), recovered from glycerol

gradients, were administered to each well in 0.8 mL of HBSS + 1% FBS. Attachment was allowed on a rocking platform at 21 °C for 60 min with a 90° change in position every 15 min. Unattached viruses were aspirated and counted in 4 mL of liquid scintillation cocktail. The plates were washed once in 0.8 mL HBSS that was counted separately. Remaining attached viruses together with cells were solubilized in 0.4 mL of HBSS containing 0.5% desoxycholate and 0.5% Triton X100. Wells were rinsed once with 0.4 mL of HBSS and combined with the cell lysate and assayed for radioactivity.

Virion protein determination

Chemical protein determinations were made according to the bicinchoninic acid assay (BCA) (Pierce) and the method described by Dorsey, McDonald & Roels (1977) with bovine serum albumin as standard and the latter with some modifications.

The reaction volume was reduced to 300 μL with 15 μL of sample, and the heating reaction was performed at 98 °C in a heating block. Microtitre plates were read at A₅₆₂ for samples assayed by BCA or at A₆₈₀ (Labsystems Multiskan[®] Multisoft) for samples analysed according to Dorsey *et al.* (1977). Protein determination by UV-absorption was made in a Nanodrop spectrophotometer (Nanodrop Technologies, Inc.), and for calculations the following equations were used: $183 \times A_{230} - 75.8 \times A_{260} = \mu\text{g protein mL}^{-1}$ (Kalb & Bernlohr 1977) and $165.6 \times A_{260} = \mu\text{g protein mL}^{-1}$ (Smith *et al.* 1969).

Enzymatic treatment of intact virions of IPNV

Purified radio-labelled virions were exposed to an O-glycosidase. Prior to enzyme treatment, virions were transferred to 50 mM phosphate buffer, pH 5.5 by the Zeba[™] Desalt Spin Column technique (Pierce). Virus corresponding to 12.7 μg of total protein was subjected to each of the following treatments: (i) pretreatment with 28 mU of neuraminidase (Sigma), followed by 4 mU of O-glycosidase from *Streptococcus pneumoniae* (Sigma) according to the manufacturer's protocol and (ii) as a negative control with no anticipated effect treated with 15.4 mU of PNGase F (Peptide N-Glycosidase F) (Sigma). O-glycosidase activity was controlled using PNP Gal- β 1,3-GalNac

(Sigma) as a substrate. As an additional control, a mock-treated sample only in 50 mM phosphate buffer and an untreated starting material stored in TNE were included. The mock-treated and the negative control PNGase F-containing samples were each incubated for a total of 5 h at 37 °C. Pretreatment with neuraminidase was allowed for 2 h at 37 °C, after which O-glycosidase was added and incubation was continued for an additional 3 h. Samples of 100 µL volumes were applied on 2 × 2 mL glycerol gradients, 10–40% (v/v) in TNE-buffer, overlaid on 0.5 mL high-density cushions of 50% relative saturation of CsCl in 40% glycerol in TNE and centrifuged at 50 000 g_{av} at 10 °C for 1 h in an SW 50.1 rotor. Tubes were punctured and 5-drop fractions were collected from the bottom. Aliquots of 100 µL from each fraction were analysed in a TriCarb® Liquid Scintillation Analyzer (Perkin Elmer). Fractions corresponding to intact and aggregated virus were assayed for infectivity as described earlier. In some experiments, viruses exposed to the various glycosidases were applied onto linear gradients of CsCl (1.30–1.40 g mL⁻¹ of CsCl) and run at 37 400 g_{av} in an SW 50.1 rotor at 10 °C overnight. Tubes were fractionated as described earlier and 100 µL from each fraction was applied to Whatman 3MM paper filter discs, air-dried, precipitated in 10% TCA overnight and rinsed in 70% ethanol. The dried filter discs were counted as described earlier.

Sodium periodate treatment of IPNV

To intact virions of IPNV, originally stored in stabilization buffer (SB; 150 mM NaCl, 1 mM MgCl₂, 5% glycerol in 10 mM Tris-HCl, pH 7.5) and transferred into 50 mM phosphate buffer, pH 5.5, sodium periodate was added to final concentrations ranging between 1 and 50 mM. To quench any potential action of appearing aldehyde groups, glycine was added to a final concentration of 0.15 M prior to the addition of NaIO₄. The oxidative reactions were stopped after 1 h of incubation at room temperature (22 °C) in the dark by the addition of sodium sulphite to yield a final concentration of 60 mM (Ogier, Michal, Thomas, Quash & Rodwell 1994). Virion samples were subsequently analysed by rate-zonal centrifugation in glycerol gradients for migrational profiles, infectivity and in some series for attachment to CHSE-214 cells.

Results

Virion quantification by spectrophotometry and chemical colorimetric assays

A UV-absorption/protein content relationship determined for reoviruses is most often employed to quantify IPNV (Smith *et al.* 1969; Dobos *et al.* 1977). We have chosen to re-evaluate this practice and have used the method of processing UV-data described by Kalb & Bernlohr (1977), and further compared such figures obtained on protein contents with data derived by the chemical colorimetric method described by Dorsey *et al.* (1977). This latter method is based on the Lowry technique (Lowry, Rosebrough, Farr & Randall 1951) and gives a close to constant value on a total nitrogen basis for most proteins assayed. However, this latter method is laborious and time-consuming, and it is not used on a routine basis. The protein contents of six separate pools of purified IPNV were based on spectrophotometric measurements (Kalb & Bernlohr 1977) and the chemical assay (Dorsey *et al.* 1977) and concordant results were obtained. Thus, a comparison of such UV-data, from 20 separate virus pools, with data obtained by the relationship described by Smith *et al.* (1969) yielded reproducibly a 3.15 ± 0.16 (SD) times higher protein content value according to the former method. Therefore, all our data on total protein contents of IPNV preparations employed in these studies are derived from calculations according to Kalb & Bernlohr (1977) yielding figures of high fidelity.

With both infectivity titres and quantitative protein determination data available, specific infectivity figures were calculated for 10 separately TCID₅₀-titrated pools yielding a mean value of $3.1 \pm 1.97 \times 10^{11}$ (SD) TCID₅₀ mg⁻¹ of virion protein. The large CV of 64% simply mirrors the level of uncertainty harboured in all methods of viral infectivity assessment.

The molecular mass of IPNV is 55×10^6 Da, and when disregarding the 8.7% contents of dsRNA in the virions, and only considering the total protein contents, a figure of 50.2×10^6 Da is arrived at. Given these data, 1 mg of IPNV protein would correspond to approximately 1.2×10^{13} virions, and subsequently, one TCID₅₀-unit in the present virus/cell system would equal approximately 40 virions of IPNV.

Glycosidase treatment of intact virions of IPNV

In this study, we wanted to investigate the possible importance of the low level of *O*-linked glycosylation of the virion capsid with regard to virion stability and viral infectivity. An initial series of experiments was performed where freshly prepared, double-radiolabelled IPNV was transferred from CsCl into a TNE buffer system and stored in this buffer at 4 °C (Dobos *et al.* 1979). Prior to enzyme treatment, virions were transferred into 50 mM phosphate buffer, pH 5.5, according to the manufacturer's protocol and then separately subjected to the enzymes PNGase F (a negative control with no expected effect), or neuraminidase followed by *O*-glycosidase (NA/O series). Following these treatments, virions were sedimented in glycerol gradients under calculated conditions where intact virions would migrate to the centre of the tubes. As controls of sedimentation, starting material in TNE and virions mock-treated only in the phosphate buffer were also included. The sedimentation profiles showed that mere transfer of virions into the acidic phosphate buffer (mock series) and the prolonged incubation at 37 °C caused the virions to disassemble as revealed by some aggregates sedimenting onto the high-density cushion and also

leaving slowly migrating capsid components on top of the gradients (Fig. 1). It also became apparent that virions stored in TNE spontaneously disaggregated as revealed by slowly sedimenting material of low molecular weight remaining on top of the gradients (Fig. 1d). Such material contained both RNA and protein of approximately the same relative amounts as in intact virions and would correspond to released core structures and capsid proteins (Hjalmarsson *et al.* 1999). Infectivity titrations were performed on pooled fractions from the virion positions in the middle of the gradients as well as from aggregated material. The same amounts of radioactivity from all series were assayed, and infectivity yields of the enzyme-treated series were compared with the TNE control and the mock series. Only minor differences were revealed, indicating no effect of the *O*-glycosidase and as anticipated also a lack of effect in the negative control series. Among the aggregates, some infectivity was detected; however, only for the negative control series. Thus, any *O*-glycosidic enzyme effect on virion stability and viral infectivity could not be deduced from this type of experimental set-up.

To further analyse the stability of the virions upon exposure to the low-pH phosphate buffer

Figure 1 Rate-zonal sedimentation in glycerol gradients of glycosidase-treated virions of infectious pancreatic necrosis virus (IPNV). Purified virions metabolically radiolabelled with ^{32}P -orthophosphate and ^3H -amino acids were stored in TNE. Virus was transferred into 50 mM phosphate buffer, pH 5.5, and 12.7 μg of IPNV was processed for each series as described in Materials and methods. (a) Mock-treated sample in 50 mM phosphate buffer, pH 5.5. (b) Virions pretreated with neuraminidase followed by *O*-glycosidase (NA/O). (c) Negative control of virions treated with PNGase F (PNGF) where no effect should be observed. (d) Starting material stored in TNE buffer. Sedimentation was from right to left.

system and after enzyme treatment, double-labelled virions were again treated as above and subsequently run in CsCl equilibrium density gradients overnight. The banding profiles revealed that whether enzyme-treated or not, the virions disaggregated severely under the high-ionic-strength conditions during the rebanding in the CsCl gradients and also yielded split peaks in the virion regions indicative of the presence of partially disrupted particles (not shown).

The fact that virions were destabilized spontaneously in the low ionic strength and pH 5 phosphate buffer prompted us to find conditions where possible enzymatic effects could be distinguished from the mere buffer effect. Therefore, the previous set of experiments with glycerol gradient analysis was repeated, now with the difference that virions were treated in a more physiological 150 mM phosphate buffer of pH 5.5, and only compared with a series after O-glycosidase treatment. The sedimentation profiles displayed symmetrical peaks of virions migrating 50% into the gradients and with some aggregates accumulating on the cushions in both series (not shown). Infectivity titrations of the same amount of radioactivity and related to the TNE control yielded infectivity data, all closely within the same orders of magnitude, and therefore, a total lack of effect following O-glycosidase treatment was again apparent.

Storage conditions promoting virion stability

We have in this laboratory noticed that IPNV stored at 4 °C in the TNE buffer system on occasion to some extent spontaneously disaggregates and loses infectivity (Fig. 1d). Therefore, it was important to keep radiolabelled viruses intact and infectious under storage conditions at 4 °C because we wanted to avoid freezing and thawing of the virus. To investigate this further, an alternative buffer system was developed. Equal amounts of freshly purified IPNV in CsCl were directly transferred into two different buffer systems, TNE and a stabilizing SB-buffer, by employing the Zeba™ Desalt Spin Column technique. The samples were subjected to 10 cycles of freeze-thawing; after each step, aliquots were removed for UV-spectrum analysis, and after steps 2, 7 and 10, samples were withdrawn for infectivity titration. UV-spectra revealed constant and overlapping profiles for virions maintained in the SB system, whereas data for virions in TNE fluctuated, indi-

Figure 2 Infectivity titrations of infectious pancreatic necrosis virus in the TNE- and stabilizing buffer-buffer systems following the cycles of freeze-thawing (F-T). Viruses were treated and analysed as described in Materials and methods.

cating difficulties in removing representative 2 µL samples for spectrometry because of possible freeze-thawing-induced aggregation (not shown). Infectivity titration data showed that after the tenth freezing, the infectivity dropped by four orders of magnitude for viruses in TNE, whereas in the SB-system infectivity was essentially maintained and was reduced only by a factor of approximately 5.5 (Fig. 2). However, as a novel observation, virions retained stability and infectivity for prolonged periods of time at 4 °C in SB as compared with the conventional TNE buffer system (compare Fig. 1d).

Glycosidase treatment of virions transiently stored in glycerol-containing buffer

The SB system was subsequently chosen and spontaneous disaggregation was not seen for viruses stored in this buffer, neither in the EM nor by rate-zonal centrifugation analyses of radiolabelled viruses. Viruses were transferred to the recommended 50 mM phosphate buffer system of pH 5.5, and one series was treated with PNGase F as a negative control with no anticipated effect and another with neuraminidase followed by O-glycosidase. As a control of mere buffer treatments, a mock-treated sample was included together with the starting material kept in SB. Both glycerol rate-zonal and CsCl isodensity centrifugations were run as described before. The sedimentation profiles of the former showed that only minor amounts of aggregates had formed and that virions migrated to the expected middle position of the centrifuge tubes

Figure 3 Rate-zonal sedimentation in glycerol gradients of glycosidase-treated infectious pancreatic necrosis virus (IPNV). Purified IPNV, metabolically radiolabelled with ^{32}P -orthophosphate and ^3H -amino acids was stored in the stabilizing buffer (SB) system. Prior to enzymatic treatment virions (12.5 μg per sample) were transferred into 50 mM phosphate buffer, pH 5.5. (a) Mock-treated series, (b) virus treated with neuraminidase followed by O-glycosidase (NA/O), (c) virus treated with PNGase F (PNGF) as a negative control and (d) starting material maintained in SB. Sedimentation was from right to left.

(Fig. 3). In the equilibrium density gradients, virions were recovered as distinct and symmetrical peaks in the centre of the tubes and neither aggregation nor disruption of the virions could be seen (not shown). Apparently, virions stored in 5% glycerol prior to transfer into the low-ionic-strength phosphate buffer protected the virions from spontaneously falling apart by the buffer treatment. Such stabilizing effect was particularly evident upon the isodensity centrifugation in CsCl. Infectivity assessment of the same amount of radioactive viruses recovered from the glycerol gradients revealed infectivity titres of the mock-treated, the negative control PNGase F and the NA/O series of 1.9×10^{10} , 3.1×10^{10} and 2.5×10^{10} TCID₅₀ mL⁻¹, respectively. Thus, the O-glycosidase enzyme treatment again showed no effect on the infectivity as compared with buffer-treated mock samples and as expected the negative control series.

Periodate oxidation of intact virions of IPNV

A series of experiments were subsequently designed to investigate the accessibility of carbohydrate residues to the chemical oxidant sodium periodate. ^3H -amino acid-labelled or ^3H -mannose-labelled viruses, stored in SB and transferred to 50 mM phosphate buffer, pH 5.5, were treated with different concentrations of sodium periodate as described in Materials and methods and were subsequently centrifuged in glycerol gradients. In a reproducible manner, it was observed that 1 mM

of periodate kept a majority of the virions intact, whereas increasing concentrations of the oxidant first caused virion disruption leaving slower migrating material on top of the gradients. This material would correspond to released core structures and VP2 as free capsomeres and complexes thereof. This is based on the fact that rate-zonal centrifugation isolation of virion cores – RNA stoichiometrically complexed with VP3 – recovered from hypotonically disrupted virions will require six times higher $g \times \text{hours}$ forces in runs on shallow sucrose gradients as compared to the present runs on steep glycerol gradients (Hjalmarsson *et al.* 1999). Then, upon exposure to concentrations between 25 and 50 mM, virions aggregated quantitatively and were recovered as fast sedimenting material on the high-density cushions of CsCl (Fig. 4). To demonstrate the reproducibility of the periodate effect, a quantification of sedimentation data from five separate experiments is summarized in Fig. 5. The positioning of columns above the indicated concentrations of periodate shows the effect of the oxidative treatment on the sedimentational behaviour of virions.

Infectivity titrations of equal amounts of radiolabelled particles migrating to the virion position in control and relevant sample gradients demonstrated that virions treated with 1 mM of sodium periodate displayed a reduced specific infectivity by three to five orders of magnitude (Table 1). In all series, where particles were recovered on the high-density cushion, no infectivity was detectable. Small amounts of intact viruses available for subsequent

Figure 4 Rate-zonal centrifugation in glycerol gradients of periodate-treated virions of infectious pancreatic necrosis virus. Purified and metabolically ^3H -amino acid-labelled virions (1.8 μg of total protein in each series) were treated with sodium periodate as described in Materials and methods. The control series contained mock-treated virions in 50 mM phosphate buffer of pH 5.5, and in the NaIO_4 series, the agent was administered at concentrations indicated in the panels. Sedimentation was from right to left. *NB:* The lower recoveries of radioactivity from the dense CsCl cushions are because of quenching by the CsCl because samples were counted directly (5 min) in liquid scintillation cocktail.

virus–cell attachment investigations showed that the dramatic reduction in infectivity upon periodate treatment was not because of the inhibition of attachment as the values of actual attachment for three periodate-treated samples were all in the same range as the corresponding controls and the mean figures – contrary to what might have been expected – actually exceeded the mean values of the control series by approximately 10%.

Binding of lectins to intact virions

Radiolabelled virions (5 μg) in PBS were treated separately for 1 h at room temperature with 10 μg each of the lectins soy bean agglutinin (SBA) and *Erythrina cristagalli* (coral tree) (ECL) (Sigma) that both recognize and bind specifically to glycosylated VP2 in lectin blots (Hjalmarsson *et al.* 1999). Upon rate-zonal centrifugation in glycerol gradients of possible lectin–virion complexes and of mock-treated virions in PBS, no differences in sedimentation behaviour could be revealed and neither could any change in infectivity be seen upon titration of recovered viruses from the gradients (not shown).

Discussion

In our quantitative studies on virion integrity following various enzymatic and chemical treatments, it was important to have reliable estimates of actual virion concentrations. Thus, it was reproducibly shown that a previous method, originally employed for quantification of reoviruses (Smith *et al.* 1969; Dobos *et al.* 1977), yielded data on a total protein content basis that were more than three times lower than the method used in this study. The UV-absorption data obtained according to Kalb & Bernlohr (1977) were further shown to produce protein content values close to the chemical method

Figure 5 Compilation of data from five separate rate-zonal centrifugation analyses of periodate-treated virions of infectious pancreatic necrosis virus. One series of ^3H -mannose-labelled (Exp. 1) and four series of ^3H -amino acid-labelled (Exp. #2–5) viruses were treated with sodium periodate as indicated and analysed as described in Materials and methods. The relative distribution of radioactivity recovered as distinct peaks or regions in each gradient is shown as columns in the figure. As an example of sedimentation, the radioactivity profile of experiment #5 is shown in Fig. 4. The following periodate concentrations were not tested: in exp. #1; 10, 25, 50 mM NaIO_4 , in exp. #2; 5 mM NaIO_4 , in exp. #3; 25 and 50 mM NaIO_4 and in exp. #4; 50 mM.

that gives equal results irrespective of the amino acid composition of the proteins analysed (Dorsey *et al.* 1977). We interpret this in favour of our approach and therefore consider UV-data processed in this manner (Kalb & Bernlohr 1977) to give close to a true total protein content of virions of IPNV.

From subsequently recovered data on virion protein determinations and TCID_{50} infectivity

titrations, figures on the specific infectivity related to total virion protein contents were determined (mean of 3×10^{11} TCID_{50} mg^{-1} protein). A ratio of virions over infectious units of approximately 40 virions/ TCID_{50} unit was also obtained in our virus/cell system.

During our initial studies on IPNV stability, we noticed that metabolically radiolabelled virions of IPNV freshly prepared in the established TNE buffer system (Chang, Macdonald & Yamamoto 1978; Dobos *et al.* 1979) displayed totally unpredictable stability properties as revealed by both rate-zonal centrifugation and by viewing in the electron microscope. These observations were consistent throughout the studies when virus preparations were stored in the TNE buffer system. Furthermore, a total disaggregation of purified virions of IPNV is provoked by dialysis against buffers of 5 mM Tris-HCl, pH 8.1 and 0.2 mM EDTA (Hjalmarsson *et al.* 1999). This behaviour could be a reflection of the biological fact that most non-enveloped viruses are characterized by their properties of metastability (Witz & Brown 2001; Johnson 2003). The human adenovirus type 2 further exemplifies the fragility of purified non-enveloped viruses. This virus disrupts readily upon dialysis against hypotonic buffers of pH < 6.5 (Prage, Pettersson, Höglund, Lonberg-Holm & Philipson 1970). This effect is partially counteracted by physiological concentrations of sodium chloride and it is almost fully inhibited in the presence of any protein, such as bovine serum albumin, that non-specifically adheres to the virions. Interestingly, because of hydrophobic interactions, these protective effects are further enhanced in the presence of non-ionic detergents such as Tween 20 (Everitt, de Luca & Blixt 1992). Not only the property to completely disaggregate under small changes of the environmental conditions *in vitro* should be considered – as seen here – but also the possibility of ‘particle breathing’, meaning slight conformational changes of the whole virion leading to the exposure of different immunological epitopes and otherwise hidden structural residues because of changes in pH and ionic strength of buffers. Such virion behaviour is seen in the poliovirus system where the N-terminal region of VP1 under certain conditions is retained at the interior of the capsid as revealed by X-ray crystallography, whereas infectious virus in suspension can be precipitated by antibodies against the N-terminal part (Roivainen, Piirainen, Rysa, Narvanen & Hovi

Table 1 Infectivity of infectious pancreatic necrosis virus (IPNV) following periodate treatment and virus re-isolation after rate-zonal sedimentation in glycerol gradients^a

Experiment	Mock-treated control	1 mM periodate-treated	Fold reduction of specific infectivity
³ H-mannose-labelled IPNV exp. #1	1.6×10^9	5.2×10^4	3.1×10^4
³ H-amino acid-labelled IPNV exp. #2	1.5×10^8	$< 10^3$	$> 10^5$
Exp. #3	3.2×10^8	7.9×10^4	4.1×10^3
Exp. #4	1.6×10^8	1.6×10^3	10^5
Exp. #5	1.6×10^8	$< 10^3$	$> 10^5$
		3.9×10^{2b}	4.1×10^5

^aThe same amounts of radiolabelled mock- and periodate-treated virions were assayed for infectivity in each separate experiment and titres are given as TCID₅₀ mL⁻¹.

^bVirions recovered after treatment with 5 mM of periodate.

1993). Further, electron microscopy of polioviruses stained with phosphotungstic acid under different temperatures and ionic concentrations displays altered diameters of the capsid, which in turn are mirrored by different periods of time needed for the chemical agent *N*-acetyl-ethyleneimine to penetrate and inactivate the virus (Burrage, Kramer & Brown 2000). The entry mechanisms of non-enveloped viruses are still quite elusive. Many non-enveloped viruses have an internal structure, often an integral part of the capsid protein, which is referred to as a membrane-active peptide. It has the equivalent function of the fusion peptides in enveloped viruses with the difference that the former cause membrane disruption to allow for virion entry and/or a simultaneous uncoating instead of membrane fusion. This membrane-active protein is hidden during the extracellular phase and needs to be activated in some viral systems by a cleavage event (Zhang, Jin, Fang, Hui & Zhou 2010), and it is exposed during the early phase of infection induced by receptor binding or alterations in the physical environment such as temperature, ionic strength or a lowering of pH (Banerjee & Johnson 2008). To a certain degree, these conformational changes are reversible and account for the observed 'capsid breathing'. A membrane-active domain responsible for membrane destabilization with subsequent entry and uncoating has not been identified for IPNV, but it is likely that such an activity would be located to VP2. It is possible that the observed virion instability in TNE could induce such 'capsid breathing' exposing a membrane destabilizing part of VP2. Further studies are needed to verify if this is the case and to identify the mechanism that allows IPNV to escape the endosomal vesicles and release the viral genome.

To prevent isolated and purified viruses of IPNV from losing infectivity and falling apart, we devel-

oped a SB for the storage of viruses both at 4 °C and frozen at -80 °C. That glycerol is a cryo-protective agent and preserves the infectivity of many viruses upon freezing is well established (Clark & Geary 1969). However, as the buffer subsequently required for optimal O-glycosidase enzymatic activity called for a change into the low-ionic-strength phosphate buffer of low pH, the SB had to be replaced prior to enzymatic exposure of virions. The Zeba™ desalt spin columns were employed for quantitative and rapid recovery of samples, and using unlabelled virions in the presence of ³H-labelled glycerol, it was shown that with a starting concentration of 5% glycerol in the SB system, the eluted sample would contain a maximum of 0.003% glycerol and now in the alternative buffer. Interestingly, virions that were transiently exposed to glycerol and then transferred to 50 mM phosphate buffer fully retained particle stability even at the extreme ionic strength conditions upon rebinding to equilibrium in CsCl (1.33 g mL⁻¹ of CsCl = 43% w/v) (Chang *et al.* 1978).

In the present investigation, mild enzymatic conditions were first employed to evaluate their possible ability to remove the low levels of carbohydrates in IPNV. This was carried out to keep virions intact and to allow for subsequent re-isolation for further analysis of virion stability and infectivity. Exposure of IPNV to 50 mM phosphate buffers demonstrated the instability of this virus when merely encountering low-ionic-strength conditions and incubations at 37 °C for 5 h. Thus, apart from following the manufacturer's recommendations, the idea was also that at this low-ionic-strength condition some sort of 'capsid breathing' could be induced, thereby exposing alternative sites for the O-glycosidase treatment. We also included the procedure of pretreating the viruses with

neuraminidase prior to exposure to the O-glycosidase. This is particularly relevant when expecting highly branched carbohydrate 'trees' that need to be 'pruned' before being exposed to the O-glycosidase that otherwise might be sterically prevented from recognizing the proper linkage to the polypeptide backbone. However, in this virus system, the level of glycosylation is extremely low (Hjalmarsson *et al.* 1999) and no such branched carbohydrates would be anticipated.

Taken together, O-glycosidase treatments of intact virions, irrespective of the ionic strength of the environment, did not affect stability properties or reduce the infectivity of recovered virions of IPNV. Moreover, only one enzyme is available that specifically targets O-linked glycosidic carbohydrates. The O-glycosidase employed here releases Ser- and Thr-linked β -Gal-(1 $\rightarrow$ 3)- α -GalNAc from glycoproteins. The glycosidic bonds connecting the carbohydrate moieties to VP2 could be of the type that cannot be recognized by the present O-glycosidase.

As VP2 of IPNV, upon SDS-PAGE analysis, will be specifically recognized by some unique lectins in blotting assays (Hjalmarsson *et al.* 1999), we allowed the lectins SBA and ECL to bind to intact radiolabelled virions. Upon subsequent rate-zonal centrifugation of such mixtures, neither aggregation nor faster migration than control virions was seen. The interpretation of all these experiments is that carbohydrate residues were not exposed on the intact virion to the extent or in such a way that enzymes could not recognize the O-linkage to the protein moiety of the VP2 capsomere, nor could free lectins bind in such a way that infectivity could be blocked or virions become aggregated.

The actual removal of the glycosidic linkages of VP2 is possible by chemical β -elimination (Hjalmarsson *et al.* 1999), but with compromised virion integrity. The lack of enzymatic effect on intact virions, as also seen by the full recognition of VP2 in subsequent lectin blots (not shown), prompted us to employ the established method of chemical oxidation of carbohydrate residues by treatment with periodate. Periodate oxidizes adjacent hydroxyl groups on carbohydrates without affecting the proteins at low pH (Woodward, Young & Bloodgood 1985; Martinez-Barragan & del Angel 2001). However, a possible effect on the amino acid residues, e.g. serine, threonine and cysteine cannot be totally excluded (Dreesman & Suriano 1976; Shraishi, Shirachi, Ishida & Sekine 1978). Pero-

date oxidation of adenovirus types 7 and 14 reduces the haemagglutinating activity against rhesus monkey red blood cells with a parallel loss of infectivity (Dreesman & Suriano 1976; Ogier *et al.* 1994). Likewise, a reduction in infectivity of more than six orders of magnitude is seen upon periodate treatment of adenovirus type 5 (Ogier *et al.* 1994). The periodate oxidation of adenovirus type 5 specifically targets the O-glycosylated fibre, but virus is still able to attach and enter permissive cells. The mechanism behind the reduction in infectivity is suggested to be the action of produced formaldehyde resulting in cross-links between the DNA and viral proteins (Ogier *et al.* 1994). Similarly, the murine cytomegalovirus is instantly inactivated by periodate, with a reduction in infectivity by 6 ¹⁰log units. However, the virus remains immunogenic resulting in the production of neutralizing antibodies in infected mice (Geoffroy, Moachon, Rodwell & Quash 1996). In our studies, we have introduced the amino acid glycine as a source of primary amino groups to possibly quench the action of exposed/released formaldehyde following the oxidative cleavage of carbohydrates. By this, we believe that the monitored effects of chemical oxidation should be a primary consequence of carbohydrate cleavage.

In conclusion, virions of IPNV were sensitive to oxidation by periodate at concentrations between 1 and 5 mM as revealed by a reduced infectivity of re-isolated and physically intact virions. The reduction in infectivity by 3–5 orders of magnitude was not a consequence of an impaired level of virus–cell attachment, but would rather reflect interference with the steps of internalization, uncoating or events further on in the replicative cycle. Higher concentrations of periodate, between 10 and 50 mM, more drastically affected virion integrity as seen by disassembly and/or formation of aggregates. We therefore suggest that the low level of O-linked glycosylation of IPNV is important for maintaining virion integrity and infectivity.

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