Subunit composition and conformational stability of the oligomeric form of the avian reovirus cell-attachment protein σC

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Previous work has shown that the avian reovirus cell-attachment sigma C (σC) protein is a multimer. In the first part of this study the oligomerization state of intracellularly synthesized σC was analysed by different approaches, including SDS–PAGE, chemical cross-linking, sedimentation and gel filtration analysis. All these approaches indicated that protein σC in its native state is a homotrimer. In the second part of the present work we investigated the effect of different factors and reagents on oligomer stability, in order to elucidate the nature of the forces that maintain the conformational stability of the homotrimer. Our results, based on the stabilizing effect conferred by reducing agents, demonstrate that the σC subunits are not covalently bound via disulfide linkages. They further suggest that the formation of an intrachain disulfide bond between the two cysteine residues of the σC polypeptide has a negative effect on oligomer stability. The susceptibility of the trimer to pH, temperature, ionic strength, chemical denaturants and detergents indicates that hydrophobic interactions contribute much more to oligomer stability than do ionic interactions and hydrogen bonding. Finally, our results also reveal that mammalian and avian reovirus cell attachment proteins follow different subunit dissociation pathways.

Introduction

Avian reoviruses are responsible for several diseases which cause considerable losses in poultry farming [for reviews see Jones (2000) and Robertson & Wilcox (1986)]. They are members of the Orthoreovirus genus, one of the nine genera of the Reoviridae family (Nibert et al., 1996; Robertson & Wilcox, 1986). Orthoreoviruses replicate in the cytoplasm of infected cells and contain a fragmented genome formed by 10 dsRNA segments, packaged within a non-enveloped double protein capsid shell 70–80 nm in diameter. The genomic segments are grouped into three classes, according to their size: large (L); medium (M) and small (S). Polypeptides encoded by the L genome segments are denominated lambda (λ), those encoded by the M segments mu (µ) and those specified by the S genes sigma (σ) (Nibert et al., 1996).

Protein σC, which is encoded by the third open reading frame of the S1 genome segment, has been identified as the cell-attachment protein because it is the only viral protein present in extracts of infected cells that is able to bind specifically to avian cells (Martínez-Costas et al., 1997; Shapouri et al., 1996). Orthoreoviruses replicate in the cytoplasm of infected cells and contain a fragmented genome formed by 10 dsRNA segments, packaged within a non-enveloped double protein capsid shell 70–80 nm in diameter. The genomic segments are grouped into three classes, according to their size: large (L); medium (M) and small (S). Polypeptides encoded by the L genome segments are denominated lambda (λ), those encoded by the M segments mu (µ) and those specified by the S genes sigma (σ) (Nibert et al., 1996).

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super helices (Shapouri et al., 1995). The coiled coil is an oligomeric protein folding motif consisting of two to five amphipatic \( \alpha \)-helices associated into a left-handed superhelix (Lupas, 1996). Recently, it has been demonstrated that both natural and recombinant \( \sigma C \) expressed \textit{in vitro} and \textit{in vivo} are multimers, and that correct oligomerization takes place in the absence of other virus-associated factors or intact cell structures. Moreover, cell-binding experiments showed that the affinity of protein \( \sigma C \) for cell receptors is exclusively associated with the oligomeric form of this protein (Grande et al., 2000).

The aim of the present study was to perform an extensive characterization of the multimeric form of protein \( \sigma C \), since a detailed knowledge of its structure should contribute to a better understanding of its various functions and should provide important information on the earliest events in avian reovirus infection.

### Methods

#### Cells, virus and antibodies. Primary cultures of chicken embryo fibroblasts (CEF) were prepared from 9- or 10-day-old chicken embryos and grown in medium 199 supplemented with 10% tryptose phosphate broth and 5% calf serum (all from Gibco BRL). Avian reovirus strain S1133 was grown in confluent monolayers of primary CEF cultures. Conditions for virus propagation, titration and purification have been described previously (Grande & Benavente, 2000; Martínez-Costas et al., 1995). Generation of rabbit polyclonal antiserum to protein \( \sigma C \) has also been described (Grande et al., 2000).

#### Expression of \( \sigma C \) protein. Expression of recombinant \( \sigma C \) (r\( \sigma C \)) in bacterial cells and purification of the protein from pelleted inclusion bodies has been described previously (Grande et al., 2000). Purified r\( \sigma C \) was desalted with a HiTrap desalting column (Amersham Pharmacia) and grown in confluent monolayers of primary CEF cultures. Conditions for virus propagation, titration and purification have been described previously (Grande & Benavente, 2000; Martínez-Costas et al., 1995). Generation of rabbit polyclonal antiserum to protein \( \sigma C \) also has been described (Grande et al., 2000).

#### Preparation of virus-free cytoplasmic extracts of avian reovirus-infected cells. CEF monolayers were incubated with 10 p.f.u. per cell of avian reovirus S1133 for 2 h at 37 °C. Non-adsorbed virus was then removed (this moment was considered time zero of infection) and cells were incubated for 16 h at 37 °C in medium 199 supplemented with 3% foetal bovine serum. Cells were lysed at a concentration of 3 \( \times \) 10^7 cells/ml in cold lysis buffer (10 mM Tris–HCl pH 8.0, containing 10 mM NaCl and 0.5% Nonidet P40), and nuclei and cell debris were removed by low-speed centrifugation. The resulting supernatant was subjected to ultracentrifugation (40 000 r.p.m. for 2 h in a Beckman SW50.1 rotor) to remove virus and particulate material, and the final supernatant was used as a source of \( \sigma C \) protein for SDS–PAGE and Western blot analysis.

#### Electrophoresis and Western blot analysis. Samples were resolved on either 10 or 12.5% polyacrylamide gels, as described by Laemmli (1970), except that in some experiments boiling of samples in loading buffer was replaced by incubation at the temperatures indicated in the Figures. For Coomassie blue staining, gels were incubated for 30 min in fixing solution (33% methanol and 10% acetic acid) containing 0.25% Coomassie blue, and subsequently destained by several washes in the fixing solution. For autoradiography, gels were incubated for 30 min in fixing solution, then dried and exposed to Agfa Curix AFW X-ray films. For Western blot analysis, proteins in unfixed gels were transferred to nitrocellulose membranes for 1 h at 100 mA in a semidry blotting apparatus (Bio-Rad). Bands of \( \sigma C \) were detected with rabbit antiserum against r\( \sigma C \) (Grande et al., 2000), using the alkaline phosphatase method (Sigma).

#### Gel exclusion chromatography. A Superoxide 12 column (Amersham Pharmacia, 3.2 × 300 mm) was equilibrated with a buffer containing 1 mM dodecyl \( \beta \)-maltoside, 100 mM NaCl and 50 mM sodium phosphate pH 8.0. Protein standards of known Stokes’ radii [ferritin (6.5 nm), catalase (5.22 nm), bovine serum albumin (4.55 nm), ovalbumin (3.05 nm) and chymotrypsinogen (2.09 nm)], as well as purified r\( \sigma C \), were dissolved in the same buffer at a concentration of 1 mg/ml and applied to the column. Elution was carried out at room temperature at a flow rate of 40 \( \mu \)l/min, and eluted samples were collected into 30 \( \mu \)l fractions.

#### Sucrose gradient sedimentation analysis. Approximately 150 \( \mu \)g of purified r\( \sigma C \) and 1 mg of molecular mass standards were applied to linear 5–20% sucrose gradients prepared in PBS containing 1 mM dodecyl \( \beta \)-maltoside. The protein standards of known sedimentation coefficients used in these experiments were: aldolase (11.30S); bovine serum albumin (4.30S); ovalbumin (3.66S) and chymotrypsinogen (2.58S). Centrifugation was carried out at 40 000 r.p.m. for 22 h at 4 °C in a Beckman SW 41 rotor, and 250 \( \mu \)l samples were then collected from the top of the tubes.

#### Molecular mass calculations. The molecular masses of the oligomeric and monomeric forms of r\( \sigma C \) were determined from the column filtration and gradient sedimentation analysis data, essentially as described previously for the mammalian reovirus protein \( \sigma 1 \) (Strong et al., 1991). Column filtration data of protein standards, expressed as a calibration curve of (\( \text{log} K_{av} \))1/2 versus Stokes’ radii (Siegel & Monty, 1966), permitted calculation of the Stokes’ radii for monomeric and oligomeric r\( \sigma C \). From the calibration curve obtained by sucrose gradient centrifugation of the standards we deduced the sedimentation coefficients for both the monomer and the oligomer. The estimated molecular masses of these proteins were then calculated using the equation \( M = \frac{\text{log} K_{av} \times N_{A}}{1 - \text{η}} \), where \( M \) is the molecular mass, \( a \) is the Stokes’ radius, \( \text{η} \) is the viscosity of the medium (value used 1), \( \text{ρ} \) is the density of the medium (value used 1), and \( N_A \) is Avogadro’s number (6.022 \( \times \) 10^23) (Siegel & Monty, 1966).

#### Chemical cross-linking. Glutaraldehyde (Sigma) was diluted to the working concentrations in PBS. A stock solution of dithiobis(succinimidyl propionate) (DSP) (Pierce) was obtained by dissolving the cross-linker in DMSO at 25 mM. Aliquots of r\( \sigma C \) were incubated for 30 min at room temperature with the indicated amounts of the cross-linkers. The reactions were stopped by adding Tris–HCl pH 7.5 to a final concentration of 50 mM, followed by a 15 min incubation at room temperature.

### Results

#### Subunit composition of the \( \sigma C \) oligomer

To initiate the structural characterization of the \( \sigma C \) multimer, we first sought to determine the stoichiometry of the oligomer, using various alternative approaches. First, we carried out a comparative electrophoretic analysis of the \( \sigma C \) protein present in: (i) purified extracts of transformed bacterial cells; (ii) purified avian reovirions; (iii) cytoplasmic extracts of
Fig. 1. SDS–PAGE analysis of protein σC. (a) Samples of purified σC protein expressed in bacterial cells (pIL-σC), purified reovirions (S1133), cytoplasmic extracts of avian reovirus-infected cells (S1133-CEF) and reticulocyte lysates containing 35S-labelled σC (CFS), as well as molecular mass markers (M) were resolved on a 10% SDS–PAGE gel, either before (lanes —) or after (lanes +) boiling for 5 min in Laemmli sample buffer. Proteins in lanes 1–3 were then visualized by Coomassie blue staining, those in lanes 4–7 by Western blotting, and those in lanes 8 and 9 by autoradiography. (b) A calibration curve, obtained by plotting the molecular masses of the protein markers against their electrophoretic mobility, was used to determine the molecular masses of the oligomeric and monomeric forms of the recombinant σC protein shown in lanes 2 and 3 of panel (a).

Fig. 2. Chemical cross-linking. Aliquots of purified rσC were incubated for 30 min at room temperature with the indicated amounts of glutaraldehyde (a) or DSP (b) and in the presence of 0.2% SDS. Samples were then analysed by 12% SDS–PAGE either before (lanes 1) or after boiling in Laemmli sample buffer, containing [panel (a), lanes 2–6; panel (b), lanes 2, 4, 6 and 8] or not containing [panel (b), lanes 3, 5 and 7] 5% βME. Protein bands were subsequently visualized by Coomassie blue staining.

Evaluation of the size of the σC oligomer was further addressed by performing chemical cross-linking assays with the purified rσC protein synthesized in bacteria. Incubation of rσC with glutaraldehyde or with the thiol-cleavable cross-linker DSP resulted in the formation of high-molecular mass products that were resistant to boiling in Laemmli sample buffer (Fig. 2a, b). At the lowest cross-linker concentration used, only one protein band, migrating slightly more slowly than the non-cross-linked oligomer, was detected (lane 3 in Fig. 2a, b); the deduced mass of this protein (115 kDa) suggests that the cross-linked oligomer is a trimer, and its slightly slower electrophoretic migration, compared to the non-cross-linked oligomer, is probably due to cross-link-induced formation of a more rigid conformation that retards its mobility. At higher cross-linker concentrations, an additional protein band of 220 kDa was detected (Fig. 2a, lanes 4–6; Fig. 2b, lanes 5 and 7), which probably reflects a trimer–dimer complex. When the disulfide bridge in DSP was cleaved, by boiling the cross-linked samples in sample buffer containing 2-mercaptoethanol...
The molecular masses of the monomeric and oligomeric forms of \( \sigma C \) were also determined by column filtration chromatography and sucrose gradient sedimentation analysis. In the chromatographic analyses the monomer eluted just before the ovalbumin marker, with an apparent molecular mass of 40 kDa and an estimated Stokes’ radius of 2.81 nm, whereas the oligomer eluted just after the catalase marker, with an apparent molecular mass of 240 kDa and an estimated Stokes’ radius of 5.23 nm. On the other hand, the results obtained after sucrose sedimentation analyses gave estimated sedimentation coefficients of \( 3.03 \times 10^{-13} \) s for the monomer, and \( 5.21 \times 10^{-13} \) s for the oligomer. Finally, when these values were combined, following the method of Siegel & Monty (1966), the molecular masses of both the monomer and the oligomer were determined to be 36 and 115 kDa, respectively. Taken together, these results demonstrate that \( \sigma C \) protein is a homotrimer.

**Stability of the \( \sigma C \) oligomer**

In the second part of this study we sought to investigate the nature of the forces that maintain the integrity of the \( \sigma C \) oligomer. To this end, we evaluated the capacity of different factors and reagents to dissociate the \( \sigma C \) multimer present in reovirions and/or in extracts of avian reovirus-infected cells.

**Temperature.** The oligomer present in extracts of avian reovirus-infected cells resisted incubation at temperatures up to 65 °C, but was completely disrupted when heated at 95 °C (Fig. 3a). The midpoint temperature (\( T_m \)) of the oligomer-tomonomer transition appears to be around 80 °C, indicating that the oligomer possesses a highly thermostable quaternary conformation. When the incubation was performed in the presence of Laemmli sample buffer, subunit dissociation of the oligomer was first detectable at 40 °C, and the \( T_m \) was reduced to 50 °C (Fig. 3b). Thermal dissociation of the trimer appears to occur as a one-step process, since no \( \sigma C \) species other than the trimer or the monomer were detected in these assays.

**Reducing agents.** The results shown in Fig. 4(a) reveal that the oligomer arrangement is disrupted upon heating reovirions in Laemmli sample buffer, irrespective of its reducing capacity (lanes 2, 4, 6 and 8). The fact that subunit dissociation of the oligomer takes place when heated in a non-reducing buffer (lanes 4 and 8) demonstrates that the \( \sigma C \) polypeptides are not linked via disulfide bonds.

Interestingly, two isoforms of monomeric \( \sigma C \) were generated when reovirions were heated in the nonreducing buffer, but only one when heated in the reducing buffer (Fig. 4a, compare lanes 2 and 4). The retarded isoform corresponds to the fully reduced \( \sigma C \) polypeptide since it comigrates with the isoform obtained upon heating reovirions under reducing conditions (lane 2). To assess whether the faster-migrating band represents a nonreduced isoform of the \( \sigma C \) polypeptide, purified reovirions were heated at 80 °C in nonreducing sample buffer, and then half of the sample was boiled in nonreducing buffer (Fig. 4b, lane 1) and the other half in a buffer containing 5% \( \beta \)ME (Fig. 4b, lane 2). A comparative SDS-PAGE analysis of the two samples revealed that \( \beta \)ME promoted the conversion of the faster-migrating band into the slower-migrating band, demonstrating that the former corresponds to a nonreduced isoform of monomeric \( \sigma C \) containing an intramolecular disulfide bond between the two cysteine residues present in the \( \sigma C \) polypeptide at positions 182 and 222.

The results shown in Fig. 4(a) also suggest that the oligomer is more stable in the presence of \( \beta \)ME, since a fraction of the multimer present in extracts of infected cells still remains undisrupted after heating in reducing sample buffer (lane 6),...
Stoichiometry and stability of oligomeric σC

Fig. 4. Effect of reducing agents on oligomer stability. (a) Aliquots of purified reovirions (S1133) and of cytoplasmic extracts of infected cells (S1133-CEF) were incubated for 5 min at either 4 or 80 °C in Laemmli sample buffer containing (lanes +) or not containing (lanes −) 5% βME. (b) Purified reovirions were heated for 5 min at 80 °C in βME-free Laemmli sample buffer, and then half of the sample was boiled in βME-free buffer (lane 1) and the other half was boiled in buffer containing 5% βME (lane 2). (c and d) Purified reovirions (S1133) and cytoplasmic extracts of infected cells (S1133-CEF) were incubated for 30 min at 40 °C in Laemmli sample buffer containing the indicated concentrations of βME (c) or DTT (d). All samples were subsequently analysed by 12% SDS–PAGE and Western blotting. The positions of molecular mass markers (expressed in kDa) are shown on the left of panels (a) and (b) and in the middle of panels (c) and (d).

but not in βME-free sample buffer (lane 8). To confirm this suggestion, we next evaluated the stability of the oligomer under different concentrations of reducing agents. As shown in Fig. 4(c), increasing the βME concentration in the Laemmli sample buffer rendered the oligomer more stable. Enhanced oligomer stability was also observed when another reducing agent, dithiothreitol, was used in place of βME (Fig. 4d).

Salts. To study the effects of salts on the stability of the σC oligomer, purified reovirions were heated at 40 °C in βME-free sample buffer containing increasing concentrations of monovalent and divalent chloride salts. As shown in Fig. 5(a), the stability of the oligomer increased when the concentration of NaCl, KCl, MgCl₂ or CaCl₂ in the sample buffer was increased. Similar results were obtained when this analysis was performed with cytoplasmic extracts of infected cells (data not shown). Furthermore, the stabilizing effect of MgCl₂ and CaCl₂ could be reversed by addition of a cation chelator such as EDTA (data not shown).

pH. The pH dependence of the stability of the σC oligomer was studied by incubating extracts of infected cells in the presence or absence of Laemmli sample buffer (Fig. 5b). In its absence, the oligomer was stable at a wide pH range; it remained undisrupted upon incubation of the extracts at 60 °C under alkaline (pH 11–6) to mild acidic (pH 5–2) conditions, but was dissociated when the incubation was carried out at pH 3. Similar results were obtained when the extracts were incubated at 40 °C in Laemmli sample buffer, although in this case partial subunit dissociation was also evident at pH 5–2.

Chemical denaturants. The effects of urea and guanidine hydrochloride, two compounds that destabilize hydrogen bonds, were also tested. The oligomer present in reovirions
Fig. 5. Effect of pH and ionic strength on oligomer stability. (a) Purified reovirions were heated for 30 min at 40 °C in βME-free Laemmli sample buffer containing the indicated concentrations of chloride salts. (b) Cytoplasmic extracts of avian reovirus-infected cells were adjusted to pH 3 with sodium citrate, pH 5–2 with sodium acetate, or pH 6, 11 or 11–6 with Tris buffer. Samples were then either heated for 30 min at 60 °C and then mixed with cold 2 × βME-free Laemmli buffer (left panel), or mixed with 2 × βME-free Laemmli sample mixture and then heated for 30 min at 40 °C (right panel). All samples were analysed by 12% SDS–PAGE and Western blotting. The positions of molecular mass markers (expressed in kDa) are shown on the left of panel (a) and in the middle of panel (b).

Discussion

Our results, based on electrophoretic, chemical cross-linking, sedimentation and gel filtration analyses, demonstrate that protein σC in its native state is a homotrimer. Data derived from different studies have also shown that several other viral cell-attachment proteins including mammalian reovirus protein σ1 (Strong et al., 1991), the adenovirus fibre (Devaux et al., 1990), the influenza virus haemagglutinin (Wilson et al., 1981), the coronavirus spike protein (Delmas & Laude, 1990), the 14 kDa protein of vaccinia virus (Vázquez et al., 1998), as well as the envelope glycoproteins of vesicular stomatitis virus (Kreis & Lodish, 1986), rabies virus (Gaudin et al., 1992) and human immunodeficiency virus (Staropoli et al., 2000), are also homotrimers, suggesting that the homotrimeric arrangement have some intrinsic significance for the virus–cell attachment process.

The fact that complete disruption of the oligomer arrangement takes place upon heating under non-reducing conditions demonstrates that its subunits are not linked via disulfide linkages, and further suggests that its subunits are held together through noncovalent interactions. On the other hand, the stabilizing effect displayed by reducing agents indicates that formation of an intrasubunit disulfide bond between cysteine residues 182 and 222 has a negative effect on oligomer stability. The reducing environment of the cell cytoplasm would probably preclude the formation of such a disulfide bond, thus enhancing the stability of the oligomer,

Detergents. The presence in σC of an extended heptapeptide repeat pattern between amino acid residues 16 to 155 suggests that hydrophobic interactions among subunits must contribute significantly to oligomer stabilization. To test this hypothesis, reovirions were incubated at different temperatures in the absence of detergent, the oligomeric arrangement remained undisrupted at temperatures up to 60 °C, and its stability was progressively reduced at higher temperatures. Increasing the concentration of SDS caused the Tm of the oligomer to be progressively reduced from 80 °C (no SDS) to 37 °C (0-3 % SDS) (Fig. 6b), suggesting that hydrophobic interactions play a major role in maintaining the integrity of the oligomer.
and probably also promoting oligomer formation. This hypothesis is coherent with our finding that, while dissociation of purified reovirions in non-reducing conditions yields both reduced and non-reduced monomer isoforms, only the fully reduced monomer is generated when a similar analysis is performed with extracts of infected cells.

Contrary to the situation reported for mammalian reovirus protein σ1 (Leone et al., 1991), the stability of oligomeric σC is not lowered in the presence of divalent cations, suggesting that inter-subunit ionic interactions between charged groups make a relatively small contribution to the conformational stability of the oligomer. Further support for this suggestion is provided by the high resistance exhibited by the oligomer towards pH-induced changes, since pH changes are known to alter the ionization state of titratable groups, leading to the formation/breakage of electrostatic interactions (Beychok & Steinhardt, 1959). The discrepancy found between mammalian reovirus σ1 and avian reovirus σC regarding the role played by inter-subunit salt bridges in oligomer stability may be a reflection of the different distribution of charged residues in their N-terminal coiled-coil regions. Ionic interactions in coiled-coils occur preferentially between position g of chain 1 and position e’ of chain 2, five residues downchain (designated i + 5’ or g–e’ ion pairs) (Dames et al., 1999; Kohn et al., 1998). Thus, whereas the deduced amino acid sequence of the heptapeptide repeat pattern of σ1 predicts the existence of six such g–e’ bridges in σ1 from mammalian reovirus type-1 and type-2, and four in σ1 from type-3 (Nibert et al., 1990), only two such bridges are predicted within the putative coiled-coil region of protein σC (Shapouri et al., 1995).

Monovalent and divalent salts not only failed to destabilize the σC oligomer, but in fact increased its stability. The similarity of the results obtained with different salts suggests that the stabilizing effect is not due to specific interactions of a particular salt with the protein. The stabilizing effect of salts can be attributed to preferential hydration of the surface of the multimer or to a decrease in the destabilizing repulsive interactions between their subunits [for reviews see Timasheff & Arakawa (1989) and Timasheff (1993)]. Since no interchain repulsions between the e’ and g positions of the σC coiled-coil

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*Fig. 6. Effect of chemical denaturants and SDS on oligomer thermostability. Purified reovirions were incubated at the indicated temperatures in the presence of 8 M urea and 6 M guanidine hydrochloride (a) or in the presence of the indicated concentrations of SDS (b). Samples were then mixed with an equal amount of cold 2 x Laemml sample buffer and analysed by 12% SDS–PAGE and Western blotting. The positions of molecular mass markers are indicated on the left.*
region were predicted, we believe that addition of salts may influence the organization of water around polar groups at the protein–water interface, thus changing the chemical potential of the dissociated subunits so as to favour the oligomer (Low & Romero, 1975). The protective effect conferred by salts could be physiologically relevant to stabilization of oligomeric proteins within the cell, where they are exposed to relatively high salt concentrations. Moreover, the intracellular salt concentration could also promote the correct assembly of the oligomeric subunits (Bonafe et al., 1991).

Using gel shift as an assay for assessing the thermal stability of oligomeric σC, we found that the midpoint of the oligomer-to-monomer transition occurs around 80 °C. This is an abnormally high Tm, since most oligomeric proteins tend to dissociate at much lower temperatures. However, high Tm values have been reported for other oligomeric proteins containing coiled-coil structures, including membrane proteins like the SKC1 potassium channel (Cortes & Perozo, 1997), glycoporhinin A (Furthmayr & Marchesi, 1976) and phospholamban (Imagawa et al., 1986), biological fibres like intermediate filaments (Steinert et al., 1984), tropomyosin (Greenfield et al., 1995) and paramyosin (Halsey & Harrington, 1973), and many viral proteins implicated in cell fusion and virus attachment. The σC oligomer was also highly resistant to thermal dissociation in the presence of both 8 M urea and 6 M guanidine hydrochloride, suggesting that it has a highly compact and stable structure. In agreement with most previously published data (Couthon et al., 1995; Gratton et al., 1992), our results confirm that guanidine hydrochloride is a more efficient denaturant than urea, as demonstrated by both the higher concentration and the lower temperature required to dissociate the oligomer. The extraordinarily high stability of oligomeric σC also indicates that subunit–subunit or quaternary interactions alone contribute significantly to maintaining the conformational stability of the multimer, by keeping the three subunits interacting together and thus protecting the tertiary conformation of its monomeric subunits, a point that may be general to many oligomeric proteins.

The presence of an extended heptapeptide repeat pattern (20 repeats) in the N-terminal region of the σC polypeptide suggests that hydrophobic interactions between subunits must play a major role in upholding the integrity of the σC oligomer. In agreement with this suggestion, the thermostability of the oligomer was greatly reduced by the presence of a relatively low concentration of SDS. Increasing the concentration from 0 to 0·3% caused a reduction in Tm of the oligomer from 80 to 37 °C. Taken together, the results of this study suggest that hydrophobic interactions are the major force contributing to the conformational stability of oligomeric σC, and that electrostatic interactions and hydrogen bonding make much less significant contributions.

Our results regarding the stability of native protein σC to thermal and pH dissociation are very similar to those previously reported for mammalian reovirus protein σ1 (Leone et al., 1991). However, the two oligomers appear to follow different dissociation pathways. Thus, while initial studies based on chemical cross-linking and electrophoretic analyses suggested that protein σ1 is a tetramer (Bassel-Duby et al., 1987), a later study showed that this protein is in fact a trimer. The anomalous behaviour of oligomeric σ1 in SDS–PAGE gels was attributed to dissociation of its C-terminal globular head, which results in a switch from a compact ‘lollipop’ native structure to a ‘hydra-like’ conformation with retarded electrophoretic mobility (Strong et al., 1991). Therefore, it appears that oligomer-to-monomer dissociation of protein σ1 occurs as a two-step process. In the first step, subunit dissociation only occurs at its C-terminal globular part, while remaining associated at its N-terminal fibrous tail. In the second step, occurring when the protein is subjected to harsher conditions, the oligomeric arrangement is completely dissociated to give individual monomers. In contrast to the σ1 situation, the results of the present study suggest that dissociation of oligomeric σC occurs as a one-step process, since no residual oligomeric σC isomers were detected after incubation of σC with cross-linkers or with Laemmli sample buffer at different temperatures. Our results suggest both that dissociation of the σC oligomer does not pass through an intermediate ‘hydra-like’ structure and that the C-terminal globular part of the avian reovirus cell-attachment protein is more compact than that of its mammalian reovirus counterpart.

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