

Key words: coronavirus/IBV/spike protein

## Coronavirus IBV: Structural Characterization of the Spike Protein

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(Accepted 23 August 1983)

### SUMMARY

The spike protein (S; surface projection) of avian infectious bronchitis virus (IBV) strain M41 comprises two glycopolypeptides, S1 (mol. wt.  $90 \times 10^3$ ) and S2 (mol. wt.  $84 \times 10^3$ ), in equimolar proportions. The apparent mol. wt. of S was calculated as  $354 (\pm 17) \times 10^3$  following co-sedimentation with catalase in sucrose gradients. Incubation of radiolabelled IBV with urea resulted in the removal of most S1, but none of S2, from the virus particle. A similar result was obtained using low concentrations of SDS, although some nucleocapsid, but not matrix, protein was also released. 2% SDS alone was as effective as 2% SDS plus 2% 2-mercaptoethanol for the separation of S1 and S2 prior to SDS–polyacrylamide gel electrophoresis. Dithiothreitol did not remove S from virions but did decrease the buoyant density of the virus from 1.18 g/ml to 1.16 g/ml, and changed the configuration of S. It is concluded that IBV S protein is an oligomer comprising two copies of each of S1 and S2, although the possibility that there are three copies of each glycopolypeptide cannot be discounted. S is attached to the membrane by S2, while S1 has little or no contact with the membrane and may form the major part of the bulbous end of S. Interpeptide disulphide bonds do not occur in S, and the association of S1 and S2 is weak.

### INTRODUCTION

Avian infectious bronchitis virus (IBV) causes economically important disease in chickens, affecting both respiratory and non-respiratory tissues (Hofstad, 1978). Like other coronaviruses the IBV virus particle contains three major protein structures, the nucleocapsid (N), matrix (M) and the spike (surface projection or peplomer) (S) proteins (for references, see Siddell *et al.*, 1982). A polypeptide of about 50000 mol. wt. (50K) forms the N protein (Macnaughton *et al.*, 1977). The M protein comprises a polypeptide of 23K which is heterogeneously glycosylated, the major glycopolypeptide being about 30K (Stern *et al.*, 1982; Stern & Sefton, 1982*b*; Cavanagh, 1983*a*). The S protein comprises two glycopolypeptides, S1 (90K) and S2 (84K), in equimolar proportions (Cavanagh, 1983*b*). Electron microscopy has shown that S protein is bulbous or 'tear-drop' shaped and approximately 20 nm long (Davies & Macnaughton, 1979; and Fig. 1). In this communication data are presented on the association of S1 and S2 with each other and with the virus envelope.

### METHODS

*Virus preparation.* [<sup>35</sup>S]Methionine-labelled virus of the M41 strain of IBV was prepared in de-embryonated chicken eggs (Cavanagh, 1981, 1983*a*) and purified in sucrose gradients containing 50 µg/ml of bovine serum albumin (BSA). Non-labelled IBV-M41 was grown in embryonated eggs and purified as described by Cavanagh (1983*b*).

*SDS–polyacrylamide gel electrophoresis (SDS–PAGE).* This was performed in slab gels containing 10% acrylamide (Cavanagh, 1981). Samples were dissociated by 2% SDS and 2% (v/v) 2-mercaptoethanol at 100 °C for 1 min. Fluorographs were prepared (Chamberlain, 1979) using Kodak XAR or Fujimex RX X-ray film.

*Preparation of S protein.* This has been described by Cavanagh (1983*b*). Briefly, non-labelled IBV-M41 was dissociated with 2% (v/v) of the non-ionic detergent Nonidet P40 (NP40) and centrifuged at 85000 *g*<sub>s</sub> for 16 h at 4 °C in a 10 to 55% (w/w) sucrose gradient in NET buffer (100 mM-NaCl, 1 mM-EDTA, 10 mM-Tris-HCl pH 7.4) containing 0.1% NP40 and 1 M-NaCl until S had travelled about one-third of the length of the tube.

*Mol. wt. estimation of S protein.* S protein was used directly from preparative gradients. Twenty-five  $\mu\text{l}$  of S protein was diluted with 75  $\mu\text{l}$  of NET buffer containing 0.1% NP40 and 5  $\mu\text{g}$  bovine catalase (Sigma). This was layered on top of a 6 ml 5 to 20% (w/v) sucrose gradient in NET buffer containing 0.1% NP40 and centrifuged in a  $3 \times 6.5$  ml MSE swing-out rotor at 4 °C for 16 h at 70000  $g_{av}$ . Fractions of 100  $\mu\text{l}$  were collected and the constituent polypeptides detected by SDS-PAGE. The gels were stained with Coomassie Brilliant Blue, destained, and the S polypeptides visualized by silver staining (Morrissey, 1981). Pre-staining with Coomassie Brilliant Blue enhanced the sensitivity of the silver stain. The mol. wt. of S was estimated using the formulae of Martin & Ames (1961).

*Treatment of virus with urea, SDS, and dithiothreitol (DTT).* Volumes of 100 or 200  $\mu\text{l}$  of [ $^{35}\text{S}$ ]methionine-labelled IBV-M41 in NET buffer containing sucrose (about 40%, w/w) and 50  $\mu\text{g}/\text{ml}$  BSA were mixed with an equal volume of urea, in 5 mM-Tris-acetate pH 7.4 containing 50  $\mu\text{g}/\text{ml}$  BSA, or SDS, in NET buffer without BSA, at twice the desired final concentration (see figure legends). Control virus was mixed with buffer only. After 1 h at 37 °C (urea) or 30 min at 25 °C (SDS) the suspensions were diluted to 750  $\mu\text{l}$  with NET buffer containing 50  $\mu\text{g}/\text{ml}$  BSA and loaded into 1 ml tubes and centrifuged in an MSE  $3 \times 6.5$  ml swing-out rotor with the appropriate adaptor at 80000  $g$  for 2 h at 25 °C. Pellets were recovered using 2% SDS and 2% 2-mercaptoethanol. For DTT treatment, [ $^{35}\text{S}$ ]methionine-labelled virus was incubated at 37 °C for 1 h with an equal volume of buffer containing DTT at twice the desired concentration. The virus was then sedimented to equilibrium in 25 to 55% (w/w) sucrose gradients at 30000  $g_{av}$  for 16 h at 4 °C.

## RESULTS

### *Molecular weight of S protein*

Because attempts to cross-link the S polypeptides with dimethylsuberimidate were unsuccessful, the mol. wt. of S was estimated by co-sedimenting S with catalase in 5 to 20% (w/v) sucrose gradients containing NP40. Electron microscopy of non-labelled S which had been concentrated by dialysis and lyophilization showed that S had formed rosettes by aggregation at their narrow hydrophobic ends, and also larger aggregates by their bulbous hydrophilic ends (Fig. 1*a, b*). Such aggregates were obviously unsuitable for mol. wt. estimations. However, electron microscopy showed that S which had been neither dialysed nor concentrated had not aggregated (Fig. 1*c, d*). Consequently, S was used direct from preparative sucrose gradients. S sedimented in one band ahead of catalase (mol. wt. 250K) (Fig. 2). No S was detected elsewhere in the gradient or at the bottom of the tube (Fig. 2). S2 stained very poorly relative to S1, as has been reported previously for purified S (Cavanagh, 1983*b*) and observed after SDS-PAGE of over 20 unlabelled preparations of IBV (D. Cavanagh, unpublished observations). A mol. wt. estimate for S of 354 ( $\pm 17$ )K was obtained from three experiments. Since S has equimolar amounts of S1 and S2 (Cavanagh, 1983*b*) and the combined mol. wt., from SDS-PAGE data, of two molecules of each of S1 and S2 is 348K, this result indicates that S comprises two molecules of each of S1 and S2. Experiments with radiolabelled S which had been concentrated gave a major peak equivalent to that obtained with non-labelled S. In addition, some S1 and S2 was present near the top of the gradient. In some preparations the amount of material in this peak was the same as in the 354K peak.

### *Selective release of S1 by urea and SDS*

After incubation of IBV-M41 at 37 °C with 2 M-urea and pelleting of the virus, most of S1 had been removed from the virus (Fig. 3). Analysis of the supernatant by electrophoresis in tube gels showed that S1 was intact and was the predominant polypeptide present. The amount of S2, N and M present in the supernatants was very similar to that from the control virus. Essentially the same result was achieved with 6 M-urea.

Results were obtained with SDS (Fig. 4) similar to those obtained with urea. The concentration of SDS that caused selective removal of S1 varied among experiments and was critical for each experiment. Thus, in the experiment illustrated in Fig. 4, 0.010% SDS removed most of S1 (Fig. 4*b*), 0.015% removed even more (Fig. 4*c*) while 0.022% SDS disrupted more than 50% of the virus particles. Inspection of Fig. 4, the results in which were confirmed by quantitative analysis of the polypeptides in the supernatants by tube gel SDS-PAGE, shows that 0.010% SDS caused the release of some N but not M polypeptide while even more N, but not M, was released by 0.015% of SDS.

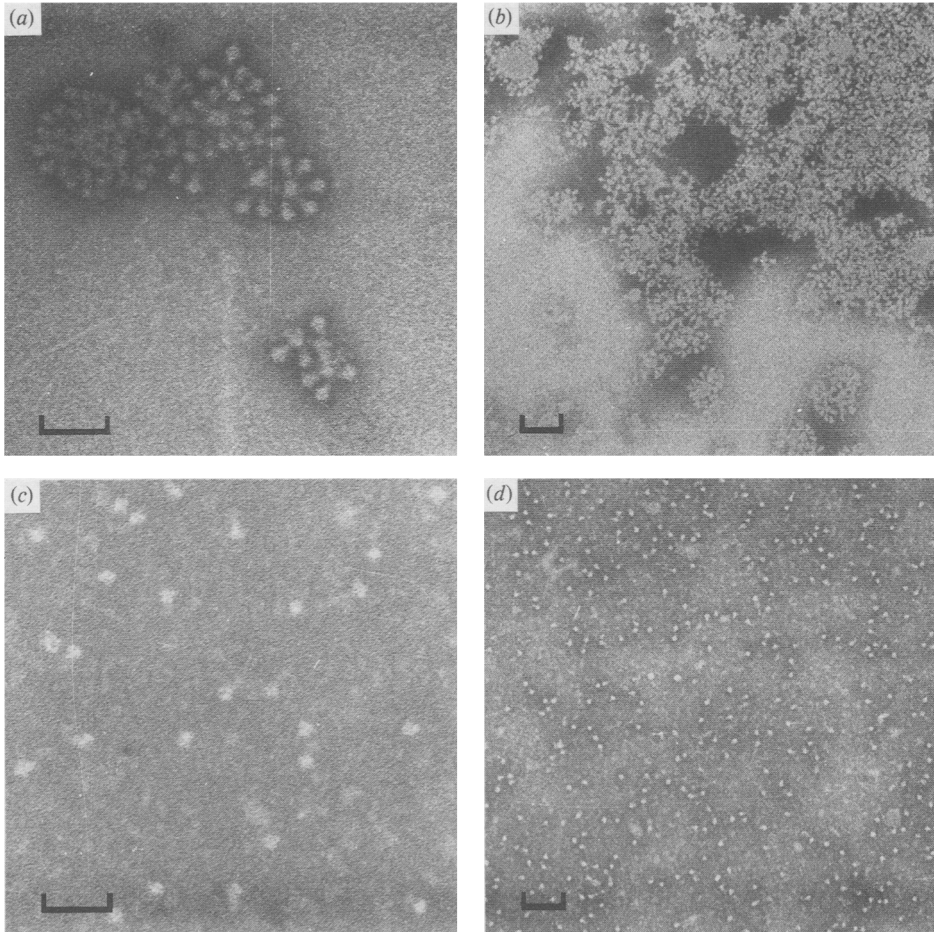


Fig. 1. Electron microscopy of purified spikes from IBV-M41. Spikes were separated from M and N protein by disruption of the virus with 2% NP40 and sedimentation in a 10 to 55% (w/w) sucrose gradient containing 0.1% NP40 and 1 M-NaCl. The spike-containing fractions of one preparation were dialysed against water containing 0.1% NP40, lyophilized and resuspended to give a 10-fold concentration (*a, b*). Another preparation of spikes was examined without prior dialysis or lyophilization (*c, d*). Bar markers represent (*a, c*) 50 nm and (*b, d*) 100 nm. The preparations were negatively stained with (*a, c, d*) 2% methylamine tungstate or (*b*) 2% potassium phosphotungstate.

These results indicate that S2 is more strongly associated with the virus membrane than S1. Indeed, S1 may not be in contact with the membrane. Electron microscopy of virus from which most S1 had been removed by 4 M-urea showed the presence of a few structures which may have been intact S; this is consistent with the SDS-PAGE finding that some S1 remained associated with the virus. Projections, thinner than intact S, were detected on some particles. These projections were variable in structure and not resolved frequently.

#### *Effect of reducing agents on IBV S protein*

IBV-M41 spikes were dissociated into their S1 and S2 components just as readily by SDS alone as by SDS with 2-mercaptoethanol at 100 °C or at 25 °C. In the absence of 2-mercaptoethanol some M did not migrate to its characteristic position; instead, some M migrated near the N polypeptide.

Incubation of [<sup>35</sup>S]methionine-labelled IBV-M41 at 37 °C with DTT resulted in a decrease in buoyant density of the virus in sucrose gradients from 1.18 g/ml to 1.16 g/ml. The percentages of

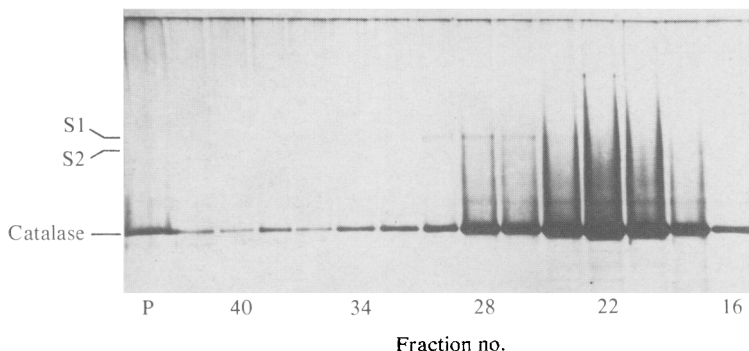


Fig. 2. Mol. wt. estimation of S from IBV-M41. Spikes were obtained as described for Fig. 1 and were not dialysed or lyophilized. S was co-sedimented with catalase in a 5 to 20% (w/v) sucrose gradient containing 0.1% NP40. Fractions of 100  $\mu$ l were collected and 40  $\mu$ l of alternate fractions analysed by SDS-PAGE followed by silver staining. Only the top part of the gel is shown. The right-hand side of the gel corresponds to the upper part of the sucrose gradient. Pelleted material (P) was resuspended in 140  $\mu$ l of 2% SDS and 2% 2-mercaptoethanol and 40  $\mu$ l analysed in the gel.

virus of density 1.18 g/ml after incubation with 0, 1, 10 and 100 mM-DTT were 82, 58, 9 and 3% respectively. SDS-PAGE showed that no polypeptides were missing from the 1.16 g/ml virus particles. Presumably the decrease in buoyant density resulted from changes in the configuration of surface proteins. After incubation at 37 °C for 1 h of purified S with 1 M-DTT, electron microscopy showed that the characteristic shape of S had gone and also that S had aggregated much more than in the control S preparation.

#### DISCUSSION

These results suggest several conclusions relating to the structure of IBV S protein. Firstly, that S is an oligomeric protein comprising two molecules of each of the glycopolypeptides S1 and S2, to give a mol. wt. of approximately 350K. Secondly, that S is a peripheral glycoprotein anchored to the membrane by S2, S1 having little or no contact with the membrane. Thirdly, that S1 is not strongly associated with S2 and that intrapeptide, but not interpeptide, disulphide bonds exist in S. Fig. 5 embodies some of these conclusions.

Ziemiecki & Garoff (1978) have shown that the spikes of Semliki Forest virus, mol. wt. 111K, bound 0.21 mg of non-ionic detergent per 1.0 mg of protein. IBV S protein had undoubtedly bound NP40 which would have led to an overestimate of the mol. wt. of S. The formulae used to calculate the mol. wt. of S apply most accurately to proteins that are essentially spherical molecules. The elongated shape of S would have reduced the sedimentation rate compared with a spherical molecule and would have resulted in an underestimate of the mol. wt. (Eason & Campbell, 1978). In one of our experiments bovine fibrinogen was co-sedimented with catalase. The estimated mol. wt. of the fibrinogen was 138K, a little less than half of the known mol. wt. of 340K. Fibrinogen is a highly elongated molecule; electron microscopy has indicated that it is at least 48 nm long with a maximum diameter of 6.5 nm (Tanford, 1961). The spikes of IBV have a mean length of 16.8 nm and a maximum width of 11.6 nm (A. Lawn, unpublished data). Since the length : width ratio for IBV S protein is considerably less than that of fibrinogen one would not expect our estimate for the amount of S to be in error by as much as that for fibrinogen, i.e. not a twofold error. Thus, it is unlikely that S comprises four copies of S1 and S2 but the possibility that there are three copies of each of S1 and S2 cannot be ruled out.

In previous work it was shown that 5 M-urea removed both S1 and S2, although a small amount of S2 remained with the virus particle (Cavanagh, 1981). The reason for the selective removal of S1 in the present report is possibly because of the presence of greater amounts of BSA which would interact with the urea. The finding that urea and SDS could separate S1 from S2 while not disrupting the rest of the virus suggests that the association of S1 and S2 is not a strong one. This is supported by our finding that some radiolabelled S had degraded to the constituent

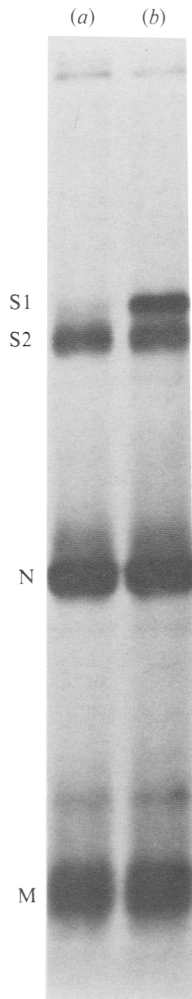


Fig. 3

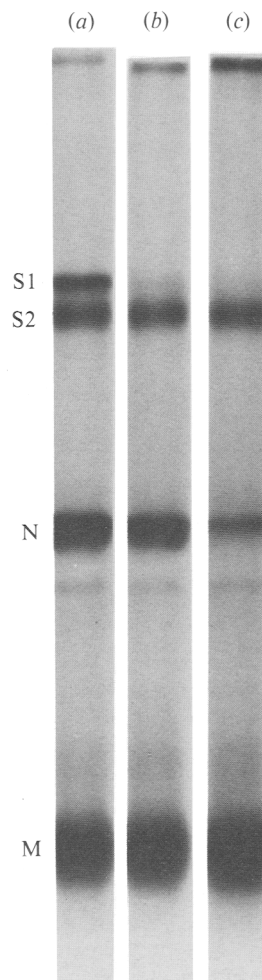


Fig. 4

Fig. 3. Selective release of S1 by urea. [ $^{35}\text{S}$ ]Methionine-labelled IBV-M41 was incubated with or without (control) 2 M-urea for 1 h at 37 °C. The virus was pelleted and then analysed by SDS-PAGE in a slab gel and radiolabel detected by fluorography. (a) Urea-treated virus; (b) control.

Fig. 4. Selective release of S1 by SDS. [ $^{35}\text{S}$ ]Methionine-labelled IBV-M41 was held at 25 °C for 30 min with or without (control) SDS. The virus was pelleted and analysed as described in Fig. 3. (a) Control; (b, c) virus after treatment with (b) 0.010% SDS and (c) 0.015% SDS.

polypeptides following dialysis and lyophilization. In addition, Stern & Sefton (1982*a*) have reported that most of the S1 of the Beaudette strain of IBV radiolabelled in chick embryo kidney cells was lost during incubation in the culture fluid. Interestingly, some N, but not M, was released by SDS treatment of IBV-M41 (Fig. 4). The non-ionic detergent NP40 can be used to release N preferentially from IBV under certain conditions (Macnaughton *et al.*, 1977; Davies *et al.*, 1981). The E2 spike glycopolyprotein of murine hepatitis virus (MHV) and bovine coronavirus L9 has covalently attached fatty acids (Niemann & Klenk, 1981; Schmidt, 1982). Our attempts to determine whether any IBV polypeptides are acylated have not been successful.

The results obtained with the reducing agents 2-mercaptoethanol and DTT indicate that S does not have interpeptide disulphide bonds but does have intrapeptide disulphide bonds, a conclusion reached for the coronaviruses porcine haemagglutinating encephalomyelitis virus

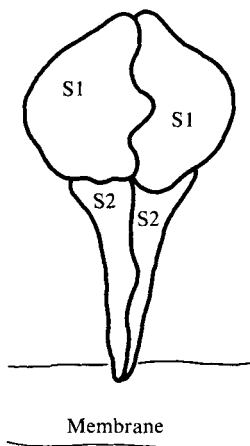


Fig. 5. A speculative model for the S protein of IBV.

(HEV) (Pocock, 1978) and murine hepatitis virus (MHV) (Sturman, 1981). Since DTT did not remove either S1 or S2 from IBV it is concluded that DTT-induced changes in the configuration of S1 and S2 were not sufficient to dissociate S1 from S2. In contrast, DTT removed two of the three high mol. wt. glycopolypeptides from HEV, and it was concluded that this was a consequence of an alteration in their configuration caused by the cleavage of intrapeptide disulphide bonds (Pocock, 1978). Also, DTT removed most of the spikes of MHV and human coronavirus 229E (Macnaughton, 1980).

I wish to thank Dr Alan Lawn of this institute for electron microscopy, and Mrs Susan Brown and Mr Philip Davis for excellent technical assistance.

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(Received 2 June 1983)