Polymeric structure of pig small-intestinal mucus glycoprotein

Dissociation by proteolysis or by reduction of disulphide bridges

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Pig small-intestinal mucus glycoprotein, of molecular weight 1.72×10^6 , is cleaved by Pronase digestion into glycoprotein subunits of molecular weight 4.5×10^5 . Of the protein component of the native glycoprotein 29% by weight was lost on Pronase digestion, with no loss of carbohydrate. The non-glycosylated region of the protein that was lost with proteolytic digestion had a broad spectrum of amino acid residues, in contrast with the glycosylated region of the protein, which was resistant to proteolysis and was rich in serine, threonine and proline residues. Reduction with 0.2 M-mercaptoethanol dissociated the Pronase-digested glycoprotein subunits into smaller glycoprotein subunits of molecular weight 2.7×10^5 . On reduction, the native glycoprotein was dissociated into subunits of molecular weight 2.4×10^5 , a similar size to those obtained from reduction of the Pronase-digested glycoprotein. On reductive dissociation of the native glycoprotein, in addition to glycoprotein subunits, protein was also released principally as a component of 90 000 molecular weight. This protein was separated quantitatively from the reduced glycoprotein in amounts compatible with one 90 000-mol.wt. protein molecule per 1.72×10^6 -mol.wt. native glycoprotein molecule. No 90 000-mol.wt. protein was released on reduction of the isolated Pronase-digested glycoprotein. Pig small-intestinal mucus glycoprotein is therefore a covalent polymer of glycoprotein subunits joined by disulphide bridges. This polymeric structure differs in important respects from that previously shown for gastric mucus, in particular with respect to the size and number of component subunits per native molecule.

Disulphide bridges and regions of protein that are accessible to proteolytic attack are an integral part of the macromolecular structure of glycoproteins from mucus secretions. The glycoprotein from pig gastric mucus, of molecular weight 2×10^6 , consists on average of four glycoprotein subunits of molecular weight 5×10^5 , and is dissociated on digestion with proteolytic enzymes (Scawen & Allen, 1977) or by reduction of disulphide bridges (Snary *et al.*, 1970; Allen & Snary, 1972). A carbohydrate-free protein of molecular weight 70 000, joined to the glycoprotein by disulphide bridges, and probably having a central structural role, is also released or digested on reduction or proteolysis respectively of the pure gastric mucus glycoprotein (Allen *et al.*, 1980; Pearson & Allen, 1980). Human gastric mucus glycoprotein is the same size and has the same type of polymeric structure as the glyco-

protein from pig gastric mucus (Pearson *et al.*, 1980). There is evidence for the importance of disulphide bridges in the structure of glycoproteins in other mucus secretions of the gastrointestinal tract (Marshall & Allen, 1978; Allen *et al.*, 1980), respiratory tract (Holden *et al.*, 1971; Roberts, 1978), the cervix (Gibbons, 1978) and the ovarian cyst (Dunstone & Morgan, 1965). However, the polymeric structure and location of the disulphide bridges in these glycoproteins remains unclear, principally because of the difficulties in isolating the undegraded glycoprotein in a soluble form and free of non-covalently bound protein or non-covalent intermolecular polymerizations. There are reports, too, of mucus glycoproteins that are not dissociated by reduction of disulphide bridges, e.g. sheep submaxillary-gland mucus, which is a polymer formed by strong non-covalent interactions between subunits of molecular weight 1.54×10^5 that are dissociated only in high salt concentrations (Hill *et*

Abbreviation used: SDS, sodium dodecyl sulphate.

al., 1977). Also, rat small-intestinal mucus (Forstner *et al.*, 1973) and human small-intestinal mucus (Jabbal *et al.*, 1976; Forstner *et al.*, 1979) are not dissociated by reduction with 10 mM-dithiothreitol in 6 M-guanidinium chloride.

It is clearly important to obtain a better appreciation of the extent and nature of the macromolecular structures of these mucus glycoproteins, particularly since the integrity of their polymeric form is essential for the gel-forming properties of mucus (Allen & Snary, 1972; Allen, 1978). In the present paper we show the undegraded glycoprotein from pig small-intestinal mucus (Mantle & Allen, 1981) to have a polymeric structure, based on interchain disulphide bridges, which is dissociated by proteolysis or reduction into subunits that are different in size and structure of those from pig gastric mucus glycoprotein.

Experimental

General methods

Details of the following are given in Mantle & Allen (1981): the isolation and purification of pig small-intestinal mucus glycoprotein; equilibrium centrifugation in a CsCl density gradient; SDS/polyacrylamide-gel electrophoresis; chemical analysis of individual amino acids and determination of monosaccharides by g.l.c. analysis; determination of sedimentation and diffusion coefficients; high-speed equilibrium centrifugation (Yphantis, 1964), and the distribution of sedimentation coefficients (Sajdera & Hascall, 1969). The diffusion coefficients for each glycoprotein preparation were independent of concentration and $D_{25,w}^0$ determined by averaging the values obtained for $D_{25,w}$ from three determinations. In all cases, measurements of the fringe counts indicated no loss of glycoprotein during ultracentrifugation.

The protein and glycoprotein contents were measured by the methods of Lowry *et al.* (1951) and Mantle & Allen (1978), with bovine serum albumin (Sigma Chemical Co., Poole, Dorset, U.K.) and purified pig small-intestinal glycoprotein respectively as standards for these assays.

The purified small-intestinal glycoprotein was kept in the following solvent systems for 48 h at 4°C before gel filtration on Sepharose 2B: 2.25 M-KCl/0.02% NaN₃; 6 M-guanidinium chloride/0.02% NaN₃; 8 M-urea/0.02% NaN₃; 1% (w/v) SDS after boiling for 2 min (with or without 0.2 M-2-mercaptoethanol); and in 10 mM-dithiothreitol/6 M-guanidinium chloride/0.1 M-Tris/HCl buffer, pH 8.5, for 4, 24 or 48 h at 22°C. Preparation of the purified small-intestinal glycoprotein for SDS/polyacrylamide-gel electrophoresis involved incubation of the material in 1% (w/v) SDS at 100°C for 2 min, both with and without 0.2 M-mercaptoethanol.

Pronase digestion of the purified pig small-intestinal glycoprotein

Pronase digestion was performed by the method of Ambler (1963) in 0.2 M-ammonium acetate/0.02% NaN₃ buffer, pH 6.5. Pure native or reduced pig small-intestinal glycoprotein (5 mg/ml) was digested with Pronase (0.4 mg/ml) for 72 h at 37°C. Further additions of the enzyme (0.2 mg/ml) were made after 24 h and 48 h. The digested glycoprotein was purified by gel filtration on Sephadex G-150 (Pharmacia, Uppsala, Sweden), when it was separated (excluded volume) from the Pronase and released peptides (included volume).

Reduction of purified pig small-intestinal glycoprotein

Purified native or Pronase-digested pig small-intestinal glycoprotein (5 mg/ml) was dissolved in 0.18 M-KCl/0.02 M-sodium barbitone/0.02% NaN₃ buffer, pH 8.6, containing 0.2 M-mercaptoethanol, and kept for 48 h at 4°C. Solid iodoacetamide was then added to a final concentration of 0.22 M, and the pH was returned to 8.6 by the addition of 1 M-NaOH. Carboxymethylation was allowed to proceed for 24 h at 4°C. After exhaustive dialysis against distilled water, the reduced carboxymethylated glycoprotein was freeze-dried.

Results

The undegraded small-intestinal glycoprotein preparation was completely excluded on a Sepharose 2B column eluted with 0.2 M-NaCl, and no lower-molecular-weight included glycoprotein was present (Fig. 1). The same elution profile was obtained after the glycoprotein had been treated with either 2.25 M-NaCl, 6 M-guanidinium chloride, 8 M-urea or boiling in 1% SDS for 2 min. The Pronase-digested glycoprotein, excluded on Sephadex G-150 and separated from the lower-molecular-weight included Pronase and peptides, when rechromatographed on Sepharose 2B was eluted as a single included glycoprotein peak, which was clearly separated from the original higher-molecular-weight excluded undigested glycoprotein (Fig. 1). Similarly, the reduced glycoprotein was also eluted from the Sepharose 2B column as a single included lower-molecular-weight glycoprotein peak. Gel filtration on Sepharose 2B thus showed that on full reduction or proteolysis of the undegraded glycoprotein a single lower-molecular-weight component was formed. However, with milder reducing conditions this was not the case; for example, reduction by dialysis against 10 mM-dithiothreitol with 6 M-guanidinium chloride for 4 h or 24 h resulted in still 60% and 40% by weight respectively of high-molecular-weight non-reduced glycoprotein being eluted in the excluded fraction (Fig. 1). Similarly, after treatment with 0.2 M-mercapto-

Fig. 1. Gel filtration on Sepharose 2B of preparations of pig small-intestinal mucus glycoprotein

A 3 mg sample of glycoprotein in 2 ml of 0.2M-NaCl/0.02M-potassium acetate/0.02% NaN₃ buffer, pH 5.5, was applied to a Sepharose 2B column (90cm × 1.5 cm) and eluted by upward flow with the same solvent. Fractions (3 ml) of the eluate were collected and analysed for glycoprotein by the method of Mantle & Allen (1978). (a) Pure glycoprotein; (b) Pronase-digested glycoprotein; (c) glycoprotein reduced by dialysis against 0.2M-mercaptoethanol for 48 h; (d) glycoprotein partly reduced by dialysis against 10mM-dithiothreitol/6M-guanidinium chloride for 4 h.

ethanol for 4 h, 60% by weight of the glycoprotein was not reduced. For complete reduction dialysis of the glycoprotein against 0.2M-mercaptoethanol for 48 h was necessary.

The values for the sedimentation coefficients and molecular weight of the different glycoprotein preparations are shown in Table 1. The $s_{25,w}^0$ values for the glycoprotein in 6M-guanidinium chloride or 2.25M-KCl were essentially the same as those previously determined (Mantle & Allen, 1981) for the undegraded glycoprotein in 0.2M-KCl/0.02M-potassium acetate/0.02% NaN₃ buffer, pH 5.5. The molecular weights in 2.25M-KCl and 6M-guanidinium chloride were also unchanged compared with those in 0.2M-KCl and indicated that no dissociation had taken place.

On sedimentation-velocity analysis the Pronase-digested glycoprotein sedimented as a single polydisperse component with an $s_{25,w}^0$ value of 10.4S. A plot of the distribution of sedimentation coefficients was polydisperse but unimodal, with a maximum $s_{25,w}$ value that lay on a plot of $1/s_{25,w}$ against C (Figs. 2 and 3). Again there was no evidence of higher-molecular-weight or lower-molecular-weight material, showing that the glycoprotein was a single component. The molecular weight, about a quarter of that of the undigested glycoprotein, was obtained by combining the $s_{25,w}^0$ and $D_{25,w}^0$ values and found to be 4.47×10^5 , whereas by the method of high-speed equilibrium centrifugation (Yphantis, 1964) the molecular weight was found to be 4.68×10^5 . Further enzymic digestion of the Pronase-digested glycoprotein did not alter the sedimentation coefficient or its molecular weight. The molecular weight of the Pronase-digested glycoprotein was also determined in 6.0M-guanidinium chloride by high-speed equilibrium concentration and was found to be 4.23×10^5 . This is in close agreement with the value obtained above for the Pronase-digested glycoprotein in the absence of 6M-guanidinium chloride,

Table 1. Physical properties of pig small-intestinal mucus glycoprotein

All values are an average of determinations on at least two different pooled preparations. All samples were centrifuged in 0.18M-KCl/0.02M-potassium acetate buffer, pH 5.5, except when the 0.18M-KCl was replaced by 2.25M-KCl or 6M-guanidinium chloride (*).

Preparation	$s_{25,w}^0$ (S)	$10^7 \times D_{25,w}^0$ (cm ² · s ⁻¹)	10 ⁻⁶ × Molecular weight	
			(s + D)	(Equilibrium)
Native glycoprotein	14.5	0.58	1.72	1.82
* Glycoprotein in 2.25M-KCl	11.8	—	—	1.86
* Glycoprotein in 6M-guanidinium chloride	14.9	—	—	1.9
Reduced glycoprotein	8.2	2.4	0.24	0.24
Pronase-digested glycoprotein	10.4	1.6	0.45	0.47
Reduced Pronase-digested glycoprotein	7.8	2.0	0.27	0.24
Pronase-digested reduced glycoprotein	7.7	2.0	0.26	0.26
Pronase-digested glycoprotein in 6M-guanidinium chloride	—	—	—	0.42

Fig. 2. Sedimentation-velocity analysis of pig small-intestinal mucus glycoprotein after reduction by 0.2 M-mercaptoethanol or digestion with Pronase

(a) ●, Native glycoprotein; ■, glycoprotein after reduction with 0.2 M-mercaptoethanol; ▲, glycoprotein after reduction with 0.2 M-mercaptoethanol and digestion by Pronase. (b) ●, Pronase-digested glycoprotein; ■, Pronase-digested glycoprotein reduced with 0.2 M-mercaptoethanol. The $s_{25,w}$ values for the appropriate glycoprotein preparations (Fig. 3) obtained from the distribution of sedimentation coefficients are shown in the open symbols.

and shows that there is no dissociation in this solvent. However, the Pronase-digested glycoprotein, when treated with 0.2 M-mercaptoethanol, was split into a single polydisperse component that sedimented with an $s_{25,w}^0$ value of 7.6 S. A plot of the distribution of sedimentation coefficients (Fig. 2) was unimodal, with no evidence of higher-mole-

Table 2. Chemical composition of the Pronase-digested pig small-intestinal glycoprotein

The monosaccharides were determined by g.l.c., and the protein content was calculated by summation of the amino acid content. The results are corrected for moisture and are averages of five determinations (except for the cysteine values, which are averages of two determinations) on independent glycoprotein preparations. For full analysis of the undegraded glycoprotein see Mantle & Allen (1981).

	Pronase-digested glycoprotein (% by weight)	Native glycoprotein (% lost on digestion)
Carbohydrate	67	1
Sulphate	3.1	
Protein	12.5	29
Moisture	18	
	(nmol/per mg dry wt. of glycoprotein)	
Sugars		
Fuc	356 ± 71	
Gal	824 ± 114	
GalNAc	1266 ± 83	
GlcNAc	674 ± 20	
Sai	746 ± 92	
Amino acids		
Lys	12 ± 1	68
His	10 ± 2	56
Arg	16 ± 3	57
Asp	19 ± 2	75
Thr	440 ± 31	0
Ser	173 ± 21	0
Glu	33 ± 3	53
Pro	257 ± 20	0
Gly	64 ± 7	31
Ala	24 ± 2	62
½Cys	39 ± 3	46
Val	80 ± 10	32
Met	10 ± 1	52
Ile	29 ± 3	44
Leu	23 ± 4	68
Tyr	12 ± 1	51
Phe	16 ± 1	52

cular-weight or lower-molecular-weight material, and the maximum $s_{25,w}$ value lay on a plot of $1/s_{25,w}$ against C for the same preparation of Pronase-digested reduced glycoprotein. The molecular weight of the Pronase-digested reduced glycoprotein was about half that of the non-reduced Pronase-digested glycoprotein, namely 2.66×10^5 from a combination of $s_{25,w}^0$ with $D_{25,w}$ and 2.37×10^5 from high-speed equilibrium centrifugation.

The chemical composition of the purified Pronase-digested glycoprotein is shown in Table 2. Although there was no significant change in the carbohydrate

Fig. 3. Distribution of sedimentation coefficients for different preparations of pig small-intestinal glycoprotein

All glycoprotein preparations were in 0.2 M-KCl/0.2 M-potassium acetate buffer, pH 5.5, sedimented at 30000 rev./min in a Beckman model E analytical centrifuge at 25°C and analysed by the method of Sajdera & Hascall (1969). The maximum $s_{25,w}$ values for each preparation are plotted on the plots of $1/s_{25,w}$ versus C in Fig. 2. (a) Glycoprotein reduced by dialysis against 0.2 M-mercaptoethanol

content, the Pronase-digested material had a protein content of 15.2% compared with 21.4% by weight for the undegraded glycoprotein (corrected to dry weight; Mantle & Allen, 1981). Since all the glycoprotein after Pronase digestion was recovered quantitatively in the Pronase-digested glycoprotein excluded on Sephadex G-150, there must have been no significant loss of carbohydrate on Pronase digestion. From the analysis of the individual amino acids in the Pronase-digested glycoprotein it is evident that the amino acids characteristic of the glycosylated region of the glycoprotein are conserved, namely threonine, serine and proline, these now comprising 69% of the total amino acids present compared with 52% in the undegraded glycoprotein. In contrast, over 30% of all the other individual amino acids are lost on Pronase digestion (Table 2). The undegraded glycoprotein contained 130 half-cystine residues per molecule, 56 of which are involved in disulphide bridges that are split on reduction. Of these half-cystine residues 55% remained after Pronase digestion (Table 2). The amount of protein removed from the undegraded glycoprotein by Pronase digestion is equivalent to 29% by weight of the total protein present.

On sedimentation-velocity analysis the fully reduced glycoprotein sedimented as a single polydisperse component with an $s_{25,w}^0$ value of 7.5 S (Fig. 2). A plot of the distribution of sedimentation coefficients was polydisperse and unimodal in shape, with no evidence of higher-molecular-weight or lower-molecular-weight material (Fig. 3), and the $s_{25,w}$ maximum lay on a plot of $1/s_{25,w}$ against C obtained from sedimentation analysis. In contrast, the distribution of sedimentation coefficients in the partially reduced glycoprotein preparations showed distinct shoulders of higher-molecular-weight material (Fig. 3). This confirms the results obtained from gel filtration of these preparations, which also showed the presence of glycoprotein in the excluded volume of the column (Fig. 1) and the presence of more than one component. The molecular weight of the fully reduced glycoprotein was 2.36×10^5 when calculated from the sedimentation coefficient and diffusion coefficient, and in good agreement was the

for 48 h (1.6 mg/ml); photographed for analysis at 1240 s after start of centrifugation. (b) Glycoprotein partially reduced by dialysis against 0.2 M-mercaptoethanol for 2 h (2.0 mg/ml); photographed for analysis at 797 s after the start of centrifugation. (c) Pronase-digested glycoprotein (1.2 mg/ml); photographed 1883 s after start of centrifugation. (d) Pronase-digested glycoprotein reduced with 0.2 M-mercaptoethanol (1.75 mg/ml); photographed 2180 s after the start of centrifugation.

value of 2.43×10^5 obtained by high-speed equilibrium centrifugation (Yphantis, 1964). These results showed that the undegraded glycoprotein was dissolved by 0.2M-mercaptoethanol into a single size of reduced glycoprotein subunit, the same size as the glycoprotein obtained by reduction of the Pronase-digested glycoprotein above. The distribution of sedimentation coefficients and the $s_{25,w}^0$ plots (Figs. 2 and 3) were in agreement for the reduced glycoprotein and the reduced Pronase-digested glycoprotein, further evidence that these two glycoprotein preparations were the same size.

The glycoprotein subunits obtained by direct reduction of the undegraded glycoprotein were digested exhaustively with Pronase, purified by gel filtration on a Sephadex G-150 column to remove the Pronase and digested peptides, and analysed by ultracentrifugation. The glycoprotein obtained sedimented as a single unimodal peak with an $s_{25,w}^0$ value of 7.85S and a molecular weight of 2.6×10^5 . These values are, within the limits of experimental error, the same as those of the reduced glycoprotein subunit before Pronase digestion, and show that the latter is not further broken down by proteolytic digestion.

After polyacrylamide-gel electrophoresis of the undegraded glycoprotein in the presence of 1% SDS under non-reducing conditions no protein bands

Fig. 4. SDS/polyacrylamide-gel electrophoresis of pig small-intestinal glycoprotein

SDS/polyacrylamide-gel electrophoresis was by the method of Weber *et al.* (1972). A 100 μ l sample of glycoprotein (5 mg/ml) was applied to the gel, and electrophoresis was for 6 h at 8 mA/gel. Protein was stained with Coomassie Brilliant Blue and the gels were scanned at 580 nm. A. Purified glycoprotein in 1% SDS (----). B. Glycoprotein reduced by incubation in 1% SDS with 0.2 M-mercaptoethanol (—). C. Pronase-digested glycoprotein reduced by incubation in 1% SDS with 0.2 M-mercaptoethanol (.....).

were visible. However, after reduction in 0.2M-mercaptoethanol a strongly staining protein band of 90000 molecular weight and weakly staining bands of 60000 and 50000 molecular weight were seen (Fig. 4). There was also a strongly staining band at the origin of the gels, where the large-size glycoprotein had not entered the gel. The protein released after reduction and carboxymethylation of the glycoprotein was separated from it by preparative equilibrium centrifugation in a CsCl density gradient (Fig. 5). Analysis of the equilibrium density gradient showed that the protein released was equivalent to $5.0 \pm 0.54\%$ by weight of the glycoprotein (five separate experiments). No protein was released when the non-reduced glycoprotein was centrifuged to equilibrium in a CsCl density gradient (Fig. 5). No protein was observed after SDS/polyacrylamide-gel electrophoresis of the reduced glycoprotein that had been separated from the 90000-mol.wt. protein fraction by the CsCl density gradient, which showed that all the 90000-mol.wt protein had been removed from the glycoprotein by this method. SDS/polyacrylamide-gel electrophoresis of the Pronase-digested glycoprotein under reducing conditions

Fig. 5. Equilibrium centrifugation of pig small-intestinal glycoprotein in a CsCl density gradient before and after reduction in 0.2M-mercaptoethanol and carboxymethylation

Glycoprotein was dissolved in CsCl at a starting density of 1.43 mg/ml: after centrifugation ($1.5 \times 10^5 g$ for 48 h at $5^\circ C$) the densities in fractions 1 and 8 were 1.38 and 1.56 g/ml respectively. Protein (Δ and $\blacktriangle$) was measured by the method of Lowry *et al.* (1951), and glycoprotein ($\square$ and $\blacksquare$) by the method of Mantle & Allen (1978). Tubes contained either native glycoprotein (Δ and $\square$) or reduced and carboxymethylated glycoprotein ($\blacktriangle$ and $\blacksquare$).

showed the absence of the 90 000-mol.wt. protein band after digestion (Fig. 4).

Discussion

The undegraded pig small-intestinal glycoprotein was shown to be free of non-covalently bound protein by SDS/polyacrylamide-gel electrophoresis (Fig. 4), by equilibrium density-gradient centrifugation in CsCl (Fig. 5) and by gel filtration (Fig. 1), and this confirmed the results obtained by Mantle & Allen (1981). Sedimentation-velocity analysis, distribution of sedimentation coefficients and gel filtration showed that the glycoprotein was a single component with the characteristic polydispersity of these mucus glycoproteins (Gibbons, 1972). The molecular weight calculated from the sedimentation coefficient and diffusion coefficient was 1.72×10^6 and by high-speed equilibrium centrifugation was 1.82×10^6 (Mantle & Allen, 1981; Table 1). The glycoprotein was not dissociated by a variety of denaturing solvents, namely 2.25 M-KCl, 6 M-guanidinium chloride, 8 M-urea or boiling in 1% SDS for 2 min, and was therefore not dissociated by disruption of non-covalent interactions alone.

On reduction the undegraded small-intestinal glycoprotein was split into a single polydisperse glycoprotein component with a molecular weight of 2.4×10^5 ($s + D$ or equilibrium; Table 1 and Scheme 1). Since the undegraded glycoprotein was shown to be a single covalent entity, it therefore consists of glycoprotein subunits joined by interchain disulphide bridges. Disulphide bridges, 28 per molecule, were present in the undegraded glycoprotein. A

protein of 90 000 molecular weight was also released after reductive cleavage of the glycoprotein, together with small amounts of proteins of 60 000 and 50 000 molecular weight that may be derived from the 90 000-mol.wt. protein, for example by proteolysis *in vivo*. The protein released on reduction was separated from the reduced glycoprotein by equilibrium density-gradient centrifugation in a CsCl gradient and amounted to $5.0 \pm 0.54\%$ by weight of the undegraded glycoprotein, which is equivalent to a molecular weight of 86 000–91 000, depending on the value of molecular weight taken for the glycoprotein. These results are compatible with each undegraded glycoprotein molecule containing one 90 000-mol.wt. protein joined to it by disulphide bridges and this protein being released concomitantly with reductive cleavage of the glycoprotein into subunits.

The Pronase-digested glycoprotein also sedimented as a single polydisperse component of molecular weight 4.5×10^5 ($s + D$) – 4.7×10^5 (equilibrium) and intermediate in size between the undegraded glycoprotein and reduced glycoprotein. On reduction this Pronase-digested glycoprotein was split into a single glycoprotein component of molecular weight 2.7×10^5 ($s + D$) – 2.4×10^5 (equilibrium), the same size as the fully reduced glycoprotein. The Pronase-digested glycoprotein was not dissociated by disruption of non-covalent linkages with 6 M-guanidinium chloride, and consequently it consists of two smaller subunits, probably joined by disulphide bridges (Scheme 1). Examination of the molecular-weight values for the different glycoprotein components suggests that the

Scheme 1. Dissociation of the native pig small-intestinal glycoprotein

undegraded glycoprotein on Pronase digestion is split into four subunits, which in turn are split into two subunits on reduction, and that the undegraded glycoprotein is composed of an average of eight of these units, all of which are about the same size (Scheme 1). Although the size of the glycoprotein subunits from Pronase digestion is one-quarter of that of the native glycoprotein, the size of the glycoprotein subunits after reduction is between one-seventh and one-eighth of the size of the native glycoprotein. This probably reflects limitations on the accuracy with which molecular weights can be determined for such large polydisperse glycoproteins. Therefore, although the number of subunits per native glycoprotein molecule appears to be eight on average, some molecules may contain fewer or even more subunits. Since the distribution of sedimentation coefficients for the reduced subunits is polydisperse, at least some, and maybe all, the polydispersity in the native glycoprotein arises from the glycosylated regions of the molecule. Differences in size and number of carbohydrate chains per glycoprotein molecule could explain such polydispersity (Gibbons, 1972).

The undegraded glycoprotein contained 21.4% by dry weight of protein, and 29% of this was lost on Pronase digestion, with conservation of the amino acids threonine, serine and proline and no loss of carbohydrate (Table 2). These results show that the protein component of the glycoprotein consists of two regions, a glycosylated region that is resistant to proteolytic attack and a region free of carbohydrate that is susceptible to proteolysis. The presence of glycosylated and non-glycosylated regions in the protein component of glycoproteins from gastrointestinal (Scawen & Allen, 1977; Allen, 1978) and other mucus secretions (Donald, 1973; Gibbons, 1978) is well documented. SDS/polyacrylamide-gel electrophoresis showed that the 90 000-mol.wt. protein, released on reductive cleavage of the glycoprotein, was absent from the Pronase-digested glycoprotein. Therefore Pronase digestion of the glycoprotein into four subunits was associated with digestion of the 90 000-mol.wt. protein. The amount of protein removed from the undegraded glycoprotein (molecular weight 1.7×10^6) by Pronase digestion is equivalent to 29% by weight of the total protein present, or to a molecular weight of about 110 000. Therefore Pronase digestion removes some further protein from the glycoprotein in addition to the 90 000-mol.wt. protein. The arrangement of the subunits in the native molecule and the role of the 90 000-mol.wt. protein require further investigation. A similar protein, but of molecular weight 70 000, has been shown to be quantitatively released on a 1:1 molar basis from pig gastric mucus glycoprotein when it is reduced. This protein, which has been shown not to be serum albumin, probably has a

central structural role in holding the subunits together by disulphide bridges (Allen *et al.*, 1980; Pearson & Allen, 1980).

The subunit structure shown in the present work for the pig small-intestinal glycoprotein is similar to that previously described for pig (Snary *et al.*, 1970; Allen & Snary, 1972; Allen, 1977) and human gastric mucus (Pearson *et al.*, 1980) glycoproteins. However, there are three clear differences between the polymeric structure of the pig small-intestinal mucus glycoprotein and those of the gastric mucus glycoproteins. Firstly, the small-intestinal mucus glycoprotein is composed of an average of eight subunits joined by disulphide bridges, not four subunits as found in gastric mucus glycoprotein. Secondly, whereas proteolysis splits both the gastric and small-intestinal glycoproteins into an average of four subunits, in the latter case the digested glycoprotein can be split further on reduction. Thirdly, the protein released on reduction is of 90 000 molecular weight in the small-intestinal glycoprotein and of 70 000 molecular weight in the gastric glycoproteins (Pearson & Allen, 1980). The small-intestinal glycoprotein is also considerably more resistant to reduction than is the gastric glycoprotein; for example, for complete reduction of the former, dialysis against 0.25 M-mercaptoethanol for 48 h is necessary, whereas the latter is dissociated by 0.01 M-mercaptoethanol for 20 h (J. P. Pearson, personal communication). Since the viscous and gel-forming properties of these mucus glycoproteins are dependent on their macromolecular polymeric structure (Allen & Snary, 1972; Allen, 1978), it is particularly noteworthy that there should be such differences in this structure for the component glycoproteins of the mucus gel from the stomach and the adjacent intestinal tract, and possibly this is related to the differences in the physiology of these two regions.

The polymeric structure of the purified pig small-intestinal glycoprotein supports previous work on pig gastric mucus that demonstrated firstly that the role of disulphide bridges in the mucus secretion involves the covalent bonding together of glycoprotein subunits to form the native glycoprotein (Snary *et al.*, 1970; Allen & Snary, 1972) and secondly that proteolysis will dissociate the native glycoprotein into its constituent subunits (Scawen & Allen, 1977). There is evidence for such structures in glycoproteins from other gastrointestinal mucus secretions, including human gastric mucus (Pearson *et al.*, 1980) and pig colonic mucus (Marshall & Allen, 1978), and pig submaxillary-gland mucus (Holden *et al.*, 1971), as well as mucus from the respiratory tract (Roberts, 1978) and cervical tract (Gibbons, 1978). Some mucus glycoproteins do not appear to possess interchain disulphide bridges; thus sheep submaxillary-gland mucus glycoprotein is a

polymer of glycoprotein subunits joined by strong non-covalent bonds that are dissociated by 2.0M-NaCl (Hill *et al.*, 1977), in contrast with the glycoproteins from pig gastric and small-intestinal mucus, which are not dissociated in high salt concentrations. The glycoproteins from rat and human small-intestinal mucus are not dissociated by incubation for 4 h in 10mM-dithiothreitol and 6M-guanidinium chloride (Forstner *et al.*, 1973, 1979). However, in our experiments with this concentration of 10mM-dithiothreitol in guanidinium chloride, 60% of pig small-intestinal mucus was completely undissociated, and there was no clear subunit peak after reduction. Therefore in pig small-intestinal glycoprotein the interchain disulphide bridges are resistant to reduction under these conditions.

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