Fragmentation and reduction of bovine secretory component

Preparation of a biologically active fragment and some evidence for a multiple-domain structure

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(Received 25 July 1984/Accepted 12 November 1984)

A tryptic fragment (A) of M_r 25 000 was prepared from bovine secretory component. The fragment binds polymeric immunoglobulin, although 9 times less effectively than secretory component on a molar basis. The fragment has four buried half-cystine residues and two exposed half-cystine residues. It gives rise to two fragments of M_r 11 000–13 000 on prolonged digestion with trypsin, and these do not bind polymeric immunoglobulin. It is proposed that fragment A consists of two immunoglobulin-like domains. Bovine secretory component was found to have 9–11 buried half-cystine residues and four exposed half-cystine residues. Reduction and alkylation of the exposed residues decreases the binding of polymeric immunoglobulin by 3-fold. Initial tryptic cleavage of bovine secretory component gives a fragment (Q) disulphide-bridged to a further fragment (T). Fragment Q is similar in size to a three-domain immunoglobulin fragment, and fragment T is similar in size to a two-domain immunoglobulin fragment. The two-domain fragment A is derived from fragment Q by further tryptic cleavage. The results are compatible with the proposal by Mostov, Friedlander & Blobel [(1984) *Nature (London)* 308, 37–43] that secretory component consists of multiple immunoglobulin-like domains. The results also indicate that optimal binding of polymeric immunoglobulin involves several domains stabilized by an exposed disulphide bridge.

Secretory component (SC) has long been known to be present in external secretions both in the free form and in association with polymeric immunoglobulins A and M (see reviews by Tomasi & Bienenstock, 1968; Lamm, 1976). It appears to be closely related to, if not identical with, the extracellular part of the epithelial-cell receptor for polymeric immunoglobulins (Brandtzaeg, 1974, 1978; Brown *et al.*, 1977; Crago *et al.*, 1978; Kuhn & Kraehenbuhl, 1979, 1981). The binding of polymeric immunoglobulin to the receptor is the first stage in the transport of IgA and IgM across epithelial cells to mucosal surfaces.

Translation of the nucleotide sequence of a cDNA clone for the rabbit receptor gave an amino acid sequence that was clearly related to those of immunoglobulins (Mostov *et al.*, 1984). It indi-

cated that the extracellular portion of the receptor, and hence secretory component, probably had several immunoglobulin-like domains (for reviews on immunoglobulin domains see Davies *et al.*, 1975; Beale & Feinstein, 1976; Amzel & Poljak, 1979). We have therefore investigated bovine SC with some of the techniques used to study immunoglobulins, such as proteolytic fragmentation and partial reduction of disulphide bridges (see reviews by Cohen & Porter, 1964; Cohen & Milstein, 1967; Metzger, 1970).

Materials and methods

Materials

Trypsin, trypsin inhibitor and dithiothreitol were obtained from Sigma Chemical Co. (Poole, Dorset, U.K.); iodo[1- 14 C]acetamide was from Amersham International (Amersham, Bucks., U.K.); unlabelled iodoacetamide and SDS were from BDH Chemicals (Poole, Dorset, U.K.); CNBr-activated Sepharose was from Pharmacia

Abbreviations used: SDS, sodium dodecyl sulphate; SC, secretory component; PBS, phosphate-buffered saline (0.15M-NaCl/10mM-sodium phosphate buffer, pH 7.2).

(Hounslow, Middx., U.K.). Other chemicals were AnalaR grade from BDH Chemicals or equivalent grade from Hopkin and Williams (Chadwell Heath, Essex, U.K.).

Preparation of bovine SC

The method of Groves & Gordon (1967) as modified by Butler (1971) was used.

Fragmentation with trypsin

Solutions of protein (0.1%) in 0.15 M-NaCl/0.1 M-Tris/HCl buffer, pH 8.0, were digested with trypsin (protein/enzyme ratio 100:1, w/w) for various times at 25, 37 and 58°C. Then trypsin inhibitor at twice the weight of that of trypsin was added, and the samples were stored at -20°C.

Reduction and alkylation

Solutions of protein (0.1%) in 0.15 M-NaCl/0.1 M-Tris/HCl buffer, pH 8.0, or in 6 M-guanidinium chloride, pH 8.0, were made 10 mM with respect to dithiothreitol and left at 20°C for 1 h. They were then made 25 mM with respect to iodo[1-¹⁴C]acetamide (1 µCi/µmol) and left for a further 1 h. Reaction mixtures were then dialysed extensively against PBS containing 0.05% NaN₃ and measured on a Packard Tri-Carb scintillation counter.

Polyacrylamide-gel electrophoresis

This was carried out in 0.1% (w/v) SDS by the method of Laemmli (1970) as modified by Richardson & Feinstein (1978), with 10% and 12% (w/v) polyacrylamide gels with a 3% (w/v) stacking gel and a discontinuous Tris/glycine buffer. Densitometric scanning of stained gels was carried out on a Helena Quick Scan Junior machine. Glycoproteins were detected with the periodic acid/Schiff reagent (Fairbanks *et al.*, 1971).

H.p.l.c.

Samples (50 µl or 200 µl) were applied to an LKB TSK G3000 SW gel-filtration column and eluted isocratically with 0.1 M-sodium phosphate buffer, pH 7.1, in Milli-Q water at 1 ml/min.

Coupling of proteins to Sepharose

The manufacturers' instructions for the use of CNBr-activated Sepharose 4B were followed.

Radio-iodination of proteins

Solutions of protein (0.1%) in 0.15 M-NaCl/50 mM-sodium phosphate buffer, pH 7.2, were treated with, per ml of solution, 100 µCi of Na¹²⁵I, 50 µl of lactoperoxidase (1 mg/ml) in PBS and 50 µl of H₂O₂ (0.015%, v/v) in PBS and left for ½ h at 20°C. Then 100 µl of 1.5 M-NaCl/100 mM-sodium phosphate buffer, pH 7.2, containing 0.5% NaN₃

was added and the sample dialysed against PBS containing 0.05% NaN₃ to constant radioactivity.

Binding of SC to protein-Sepharose

The method of Socken & Underdown (1978) was followed, with some modification. Portions (100 µl) of a 30% (v/v) suspension of IgM-, IgG- or bovine serum albumin-Sepharose in PBS were added to serial dilutions of bovine ¹²⁵I-SC in a total volume of 400 µl of PBS and gently rotated for ½, 1 and 2 h at 20°C. The total radioactivity of each sample was then measured on a Packard Auto-gamma scintillation counter. The protein-Sepharose was then gently spun down, and the pellet washed four times with 1 ml of PBS and its radioactivity counted. Binding was expressed as the percentage of the total radioactivity remaining on the washed pellet.

Inhibition of ¹²⁵I-SC binding

The procedure was similar to that described by Socken & Underdown (1978). A predetermined standard amount of bovine ¹²⁵I-SC was added to serial dilutions of inhibition protein in a total of 400 µl of PBS. Then 100 µl of a 30% (v/v) suspension of human monoclonal IgM-Sepharose was added to each of the samples, which were rotated at 20°C for 1 h. Tubes containing only the standard amount of ¹²⁵I-SC and IgM-Sepharose were also included. Protein-Sepharose was then gently spun down and washed four times with 1 ml of PBS containing 0.1% bovine serum albumin, and the radioactivity of the pellets was counted. The percentage of the radioactivity of the standard pellets remaining on the sample pellets was calculated and used to obtain the percentage inhibition. This was plotted against the amount (µg) of inhibiting protein, and the amount required for 50% inhibition was used as the basis for comparison of different proteins.

Results

Binding of ¹²⁵I-SC to protein-Sepharose

No significant binding to bovine IgG or albumin coupled to Sepharose was observed. Bovine IgM-Sepharose was approximately twice as effective as human IgM-Sepharose. Because of the ready availability and purity of the human monoclonal IgM, this was used coupled to Sepharose in routine assays. A 50% inhibition was given by the following amounts: bovine IgM, 8 µg; sheep IgM, 13 µg; human IgM, 20 µg; rabbit IgM, 53 µg; horse, pig and mouse IgM, > 300 µg. Fig. 1 shows that 2.9 times the amount of reduced and alkylated bovine SC compared with that of untreated SC was required to produce 50% inhibition of ¹²⁵I-SC binding to IgM-Sepharose. This result was readily

reproducible to within 15%. It suggests that cleavage of disulphide bridges of bovine SC in the absence of guanidinium chloride affects the binding site.

Half-cystine residues in bovine SC

Upon reduction in aqueous buffer and alkylation with iodo[1-¹⁴C]acetamide bovine SC incorporated about twice the radioactivity per mol as did a Fab μ fragment treated the same way at the

same time (Table 1). Since the Fab μ fragment is known to have two accessible half-cystine residues under these conditions (Miller & Metzger, 1966; Beale & Feinstein, 1969; Beale & Buttress, 1969), the labelling results show that bovine SC has four half-cystine residues. No significant radioactivity was incorporated into bovine SC or Fab μ fragment without prior reduction (Table 1).

Upon reduction in 6M-guanidinium chloride and alkylation with iodo[1-¹⁴C]acetamide bovine SC incorporated 1.3–1.5 times the radioactivity per mol as did Fab μ fragment treated the same way at the same time (Table 1). Fab μ fragment is known to have a total of ten half-cystine residues that are accessible under these conditions. Therefore the labelling results indicate that bovine SC has a total of 13–15 of these residues. Nine to 11 of these are inaccessible in aqueous solution, as would be expected if bovine SC had a multiple immunoglobulin-like domain structure.

Fragmentation of bovine SC

Fig. 2 shows a typical h.p.l.c. separation of a 37°C tryptic digest. A peak (O), eluted at the same position as SC, decreases with time, while other peaks (A, B and C) increase. Therefore the first impression was that SC was being fragmented into several components that were reasonably stable.

A larger-scale 20min digest was then prepared and six h.p.l.c. runs were carried out at maximum loading. Pools of peaks O, A and B plus C were

Fig. 1. Inhibition of binding of ¹²⁵I-SC to human IgM-Sepharose by SC, reduced and alkylated SC and SC fragments

For experimental details see the text. ▼, SC; ●, h.p.l.c. peak O; ■, reduced and alkylated SC; ▲, fragment A; ◆, fragments B and C.

Table 1. Alkylation of reduced and non-reduced samples with iodo[1-¹⁴C]acetamide

For experimental details see the text.

Sample	Amount		Radioactivity (c.p.m.)	Sp. radioactivity (c.p.m./nmol)	Ratio of sample/ Fabμ sp. radioactivities
	(μg)	(nmol)			
Non-reduced (guanidine absent)					
Fabμ	32	0.71	211	298	—
SC	53	0.72	307	426	—
Fragment A	44	1.76	240	136	—
Reduced (guanidine absent)					
Fabμ	32	0.71	2028	2856	—
			2606*	3107*	—
SC	53	0.72	4348	6038	2.1
			4250*	5903*	1.9*
Fragment A	44	1.76	3518	1999	0.7
			4920*	2796*	0.9*
Reduced (6M-guanidine present)					
Fabμ	16	0.35	4698	13423	—
			5257*	15020*	—
SC	26	0.36	7248	20134	1.5
			7145*	19849*	1.3*
Fragment A	22	0.88	7087	8054	0.6
			8539*	9704*	0.6*

* Repeat experiment with a different batch of iodo[1-¹⁴C]acetamide.

Fig. 2. H.p.l.c. of 37°C tryptic digests of SC. For experimental details see the text; 280nm profiles are shown.

concentrated and re-subjected to the h.p.l.c. The pool of peak O was complex, the pool of peak A gave a single symmetrical peak and the pool of peak B plus C gave a double peak. Samples of the pools were reduced in the presence of SDS and subjected to SDS/polyacrylamide-gel electrophoresis (Fig. 3a). As expected, peak O gave multiple bands, peak A gave a single band and peak B plus C gave two bands.

The ability of these components to inhibit SC binding was then tested. Fig. 1 shows that on a weight basis peak O was about half as effective as SC in inhibiting the binding of ^{125}I -SC to IgM-Sepharose, peak A was about one-third as effective and peaks B plus C did not inhibit. Again this result was readily reproducible.

Characterization of the inhibiting fragment peak A

After reduction of peak A in the presence of SDS followed by SDS/polyacrylamide-gel electrophoresis, a single band was obtained lying between the position of L-chain (two immunoglobulin domains) and Fd μ (two immunoglobulin domains plus carbohydrate). An M_r of 25000 was calculated.

Further digestion of the fragment from peak A with trypsin gave two bands on SDS/polyacrylamide-gel electrophoresis, both of which ran near to the position of β_2 -microglobulin (one immunoglobulin-like domain) and were calculated to have M_r values in the range 11000–13000.

After reduction in aqueous buffer and alkylation with iodo[1- ^{14}C]acetamide the fragment A incorporated 0.7–0.9 times the radioactivity per mol as did Fab μ fragment treated the same way at the

same time (Table 1). This showed that there were two accessible half-cystine residues. There was no significant labelling without reduction.

After reduction in 6M-guanidinium chloride and radioalkylation the fragment incorporated 0.6 times the radioactivity per mol as did Fab μ fragment treated the same way (Table 1). Since under the conditions used Fab μ is known to have a total of ten half-cystine residues susceptible to reduction and alkylation, the labelling results show that fragment A has a total of six of these residues.

These results suggest that fragment A is a single piece of polypeptide chain folded into two immunoglobulin-like domains.

Fragmentation process

Fig. 3(a) shows the densitometric scan of an SDS/polyacrylamide gel of reduced samples of a 37°C tryptic digest of bovine SC. There are some important points to notice. A band corresponding to fragment A appears and increases at stages of the digest where there is very little or no material in the position of SC. Hence fragment A is not derived directly from SC. Instead, it would appear to be derived from a component represented by band R, which is very close to the position of an Fd μ marker (three immunoglobulin domains plus carbohydrate).

The origin of band R is less clear, but its pattern of appearance on the gel suggests that it is derived from another component represented by band Q rather than directly from SC. Fragment Q has a slightly larger M_r than fragment R but still runs very near to the position of the three-domain Fd μ marker. Q must be produced directly from SC since there are no intermediary bands of significance. Another band T appears at the same time as band Q and increases with it, although never attaining the same significance. Band T is near the position of an L-chain marker (two domains). A minor band S appears and increases during the early part of the digestion, but never reaches major proportions. It is probably produced by a slightly different pathway to the major one that gives rise to the other fragments. The periodic acid/Schiff reagent showed that bands SC, Q, R and A were glycosylated.

Non-reduced digest samples were also subjected to SDS/polyacrylamide-gel electrophoresis (Fig. 3b). There is a band R that appears, increases and decreases in exactly the same manner as band R in Fig. 3(a) and is assumed to be the same component. Hence fragment R does not require disulphide cleavage before it can be released. Similarly there is a band A, corresponding to fragment A, that behaves in exactly the same manner as band A in Fig. 3(a). Therefore the release of fragment A does not require disulphide cleavage.

Fig. 3. Densitometric scans of SDS/polyacrylamide-gel electrophoretograms of (a) reduced samples of h.p.l.c. pools and 37°C-tryptic-digestion series and (b) non-reduced samples of 37°C-tryptic-digestion series

For experimental details see the text. Abbreviations: BSA, bovine serum albumin; OVA, ovalbumin; β_2 M, β_2 -microglobulin.

There are, however, no bands in Fig. 3(b) corresponding to bands Q and T in Fig. 3(a). Instead, there is clearly much more material lying in the position of SC. The inference is that some of this material consists of fragment T disulphide-bridged to fragment Q and would therefore be the same size as SC. A minor band P is also apparent in Fig. 3(b), but never reaches major proportions. It is probably produced by a slightly different pathway to the major one that gives rise to fragments R and A.

The inter-relationship between the bands observed on electrophoresis of reduced and non-reduced samples on SDS/polyacrylamide gels was confirmed by running a two-dimensional gel as described by Allore & Barber (1983). The results from a 15 min digest are shown in Fig. 4. Some of the material lying in the position of SC in the first (non-reduced) dimension gives rise to bands Q and T in the second (reduced) dimension. Bands A, B, C and R in the first dimension are not significantly altered in the second dimension.

Fig. 4. Densitometric scans of two-dimensional SDS/polyacrylamide-gel electrophoretograms of 15 min digest of SC

For experimental details see the text. (a) First (non-reduced) dimension; (b) second (reduced) dimension.

Some of the material in the minor band P appears to give rise to the minor fragment S in the second dimension.

The most likely explanation of all these fragmentation results can therefore be summarized as follows. SC is first cleaved into a three-domain fragment Q disulphide-bridged to a two-domain fragment T. Further digestion then releases a three-domain fragment R, which is only slightly smaller than Q. Fragment R then gives rise to a two-domain fragment A and a one-domain fragment C. Prolonged digestion cleaves fragment A into two one-domain fragments, one of which is fraction B. Scheme 1 is a schematic representation of this major fragmentation pathway.

Discussion

Translation of the nucleotide sequence for a cDNA of the rabbit poly-immunoglobulin receptor (Mostov *et al.*, 1984) gives an amino acid sequence that suggests that the extracellular part, and presumably SC, might contain five immunoglobulin-like domains. The results described in the present paper confirm this suggestion. We find that bovine SC has nine to 11 buried half-cystine residues, which is very close to the expected number of ten. Further, we show that a fragment

Scheme 1. Schematic representation of one of several possible models for the main fragmentation pathway of bovine SC. The black oblong boxes represent putative immunoglobulin-like domains. Key: t, tryptic cleavage; r, reductive cleavage; -S-S-, exposed disulphide bridge. The positions of the exposed disulphide bridges are purely speculative, but there is evidence that one links a three-domain fragment (Q) to a two-domain fragment (T), and that the other lies on one of the domains of fragment A.

(A) of two-domain size has four buried half-cystine residues.

Our fragmentation results are also compatible with a five-domain structure. We obtained fragments of three-domain, two-domain and one-domain size and showed that the initial product consisted of a three-domain fragment (Q) disulphide-bridged to a two-domain fragment (T) (see Scheme 1). Some of these fragments are probably equivalent to those obtained from human SC by Kobayashi *et al.* (1973).

Our results also show that bovine SC has four exposed half-cystine residues, two of which must provide the disulphide bridge that joins the three-domain (Q) and two-domain (T) fragments. Reduction in the absence of guanidinium chloride and alkylation significantly lowers the ability of bovine SC to bind polymeric immunoglobulin. Further, although we find that the two-domain fragment A binds polymeric immunoglobulin, it is 9-fold less effective than SC on a molar basis. It therefore appears that optimal binding requires the presence of several domains whose relative orientations are stabilized by a disulphide bridge.

There appears to be some species difference in the number of exposed half-cystine residues. We obtained four for bovine SC; Cunningham-Rundles & Lamm (1975) found only two in human SC, and six might be present in rabbit SC (Mostov *et al.*, 1984).

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