A Sialoglycopeptide from Human Erythrocytes with Receptor-like Properties for Encephalomyocarditis and Influenza Viruses

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SUMMARY

Encephalomyocarditis and influenza viruses attach to human erythrocytes causing haemagglutination. The receptor for both viruses on these cells is the major membrane sialoglycoprotein, glycophorin, solubilized preparations of which inhibit haemagglutination by either virus. We show here that glycophorin preparations inhibited haemagglutination of both viruses, even after the preparations were digested with chymotrypsin. To determine which component(s) in the digest exhibited activity, peptides separated by gel filtration were assayed for haemagglutination inhibition; one peptide only, CH-0, was active. A tentative structure was deduced for CH-0 from amino acid and sialic acid analyses. It was already known that neuraminidase treatment of erythrocytes or glycophorin prevents interaction with either virus, suggesting that sialic acid may form part of the active binding site in the receptor. However, receptor activity requires more than the presence of a particular arrangement of sialic acid since the arrangement in CH-0 was identical to that in two other inactive chymotryptic peptides. Examination by gel filtration, sucrose density gradient centrifugation and SDS–polyacrylamide gel electrophoresis demonstrated that CH-0 readily aggregated, unlike the inactive peptides. It was proposed that the CH-0 chymotryptic peptide showed receptor-like activity (inhibited haemagglutination) because its tendency to aggregate allowed strong multivalent binding with virus particles.

INTRODUCTION

Glycophorin, the major sialoglycoprotein in the human erythrocyte surface membrane (Fairbanks et al., 1971; Marchesi et al., 1976), can serve as a receptor for both influenza virus (Kathan et al., 1961; Marchesi & Andrews, 1971) and for encephalomyocarditis (EMC) virus (Enegren & Burness, 1977). We have shown previously that tryptic digests of glycophorin interacted with both viruses as efficiently as undigested glycophorin (Burness & Pardoe, 1981). This suggested that fragments of glycophorin can behave as soluble receptors and gave rise to the hope that characterization of the fragments would bring closer an understanding of the fine structure of the active site for virus binding. The present investigation was undertaken to identify the active fragment(s) in glycophorin digests. Chymotrypsin rather than trypsin was used to generate fragments since chymotryptic peptides of glycophorin differ more from each other in size and are easier to separate than their tryptic counterparts (Tomita et al., 1978).

METHODS

EMC virus (K2 strain), influenza virus (PR-8 strain) and glycophorin. These were prepared and purified as described previously (Burness & Pardoe, 1981). When necessary, glycophorin was made radioactive using [3H]-acetic anhydride (Montelaro & Rueckert, 1975) or 131I-iodinated by the chloramine T procedure (Greenwood et al., 1963).

Chymotrypsin digestion of erythrocytes. Human type O red cells were washed with 'isotonic' buffer (0.15 M-NaCl containing 0.005 M-Na2HPO4 adjusted to pH 8 with H3PO4) then distributed in volumes containing 1 ml of packed cells. These cells were incubated at 37 °C with 0.4 ml isotonic buffer in the presence or absence of 2 mg chymotrypsin [N-α-p-tosyl-L-lysyl chloromethyl ketone (TLCK)-treated; Sigma] for various lengths of time after
which the cells were collected by centrifugation. The supernatant fluids containing released sialoglycopeptides were kept for sialic acid assays (Warren, 1959). The cells were used to measure virus binding and to make membranes for analysis by SDS-gel electrophoresis and for sialic acid assays.

**Chymotrypsin digestion of glycophorin.** Preparations of glycophorin containing 5 to 10 mg protein/ml in 0·1 M-phosphate buffer pH 7-5 were incubated at 37 °C with chymotrypsin (TLCK-treated) at a protein to enzyme ratio of 20:1. Digests for gel filtration were incubated for 18 h after which they were frozen and thawed and the precipitate formed removed by low-speed centrifugation. In experiments to measure the effect of time of incubation with chymotrypsin on haemagglutination inhibitory properties of glycophorin, incubation was for various lengths of time after which biological activity was measured without the freezing and thawing steps.

**Polyacrylamide gel electrophoresis.** Membranes and glycophorin preparations were examined on 5-6% polyacrylamide gels in the presence of 1 % SDS (Fairbanks et al., 1971). Chymotryptic digests were also examined on 12·5% gels containing 8 M-urea and 0·1 % SDS (Swank & Munkres, 1971). After electrophoresis, both gel types were stained with periodic acid-Schiff’s reagent (PAS) as described previously (Fairbanks et al., 1971) and scanned at 550 nm in a Gilford spectrophotometer fitted with a gel transport accessory. Apparent mol. wt. of the sialoglycopeptides were estimated on the 12·5% gels by comparison of their mobilities with those of the following markers: ovalbumin, pepsin, soybean trypsin inhibitor, trypsin, myoglobin, lysozyme, RNase A and cytochrome c.

**Gel filtration.** This was carried out on 1·5 x 90 cm columns of Sephadex G-150 superfine (Pharmacia) in 0·1 M-ammonium acetate pH 6·8 at room temperature (Tomita et al., 1978). Columns were run at 3 ml/h and the absorbance of the 1 ml fractions collected was measured at 230 nm before 50 μl samples were taken for radioactivity determinations. Fractions comprising the peaks were combined, dialysed against water, lyophilized and then redissolved in water before measuring protein (Lowry et al., 1951) and sialic acid content (Warren, 1959), amino acid composition, haemagglutination inhibitory properties and examining by sedimentation on sucrose gradients.

**Amino acid analyses.** These were performed in a Beckman Model 121 MB analyser on samples containing 0·45 to 0·75 mg protein hydrolysed at 110 °C for 24 h in 6 M-HCl.

**Haemagglutination (HA) inhibition.** Serial twofold dilutions of glycophorin or its chymotryptic peptides containing 200 μg protein/ml in phosphate-buffered saline (PBS) were made on microtitre plates. To each microtitre well was then added an equal volume (50 μl) of either EMC or influenza virus (8 HA units) followed by 50 μl 0·2% human red blood cells in 50% PBS containing 0·1% gelatin–50% 4·5% (w/v) glucose. Endpoints, recorded after keeping the microtitre trays at 4 °C overnight, were used to calculate the number of HA units of virus inhibited by 1 μg protein.

**Sucrose gradient sedimentation.** Samples were centrifuged on 10 to 30% sucrose gradients in 0·02 M-phosphate buffer pH 8 containing 0·1 M-NaCl and also, in some experiments, 0·1% SDS, in a Beckman SW50·1 rotor at 50000 rev/min for 6·5 h or, in SDS, for 22 h. Each tube contained 10 mg bovine serum albumin as a marker. Fractions of about 0·3 ml were collected, diluted with water to 1 ml for absorbance measurements at 280 nm and for radioactivity measurements.

**RESULTS**

**Effect of chymotrypsin on erythrocytes**

Incubation of human erythrocytes with trypsin has no effect or enhances their haemagglutination by EMC or influenza viruses (Burness & Pardoe, 1981; Enegren & Burness, 1977). To determine whether this was also true of chymotrypsin, human erythrocytes were incubated in the presence or absence of chymotrypsin for various lengths of time. The amount of sialic acid in the sialoglycopeptides released into the supernatant fluid was used to follow the course of the digestion of the sialoglycoprotein in the erythrocyte surface. Assuming that hydrolysis of membranes with 0·05 M-H₂SO₄ at 80 °C for 1 h releases 100% of the sialic acid (Burness & Pardoe, 1981), incubation with chymotrypsin released 38% of the total sialic acid after 1 h, 48% after 2 h and 73% after about 18 h (Fig. 1).

Despite the loss of sialic acid from the cell surface, there was little effect on virus attachment to the cells which was measured in two ways. In the first method, the enzyme-treated erythrocytes were themselves used for haemagglutination assays. This method gave identical HA titres for erythrocytes incubated in the presence or absence of chymotrypsin whether EMC or influenza virus was tested, even after overnight incubation of cells with enzyme (result not shown). In the second method, enzyme-treated and control cells were incubated with EMC or influenza virus for 1 h to allow virus to adsorb; the cells were collected by centrifugation and the
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Fig. 1. Effect of chymotrypsin on erythrocytes. Cells were incubated with chymotrypsin for various lengths of time, collected by centrifugation and the sialic acid in the supernatant fluid (■) measured after acid hydrolysis. The treated cells were then used to measure their ability to adsorb EMC virus (●) or influenza virus (○).

![Graph showing the effect of chymotrypsin on virus binding and sialic acid release.](image)

Fig. 2. Effect of chymotrypsin on the SDS-polyacrylamide gel electrophoretic profiles of erythrocyte membranes and glycophorin. Membranes were prepared from erythrocytes incubated in (a) the absence or (b) the presence of chymotrypsin for 5 min. Glycophorin was incubated in (c, e) the absence, or (d, f) the presence of chymotrypsin for (d) 1 h or (f) 4 h. Samples were analysed on (a to d) 5-6% or (e, f) 12-5% gels which were stained with PAS reagent.

![Electrophoretic gels showing changes in protein profiles.](image)

loss of virus from the supernatant fluid was measured by haemagglutination assays using untreated cells. This second procedure showed that cells incubated with chymotrypsin were as effective as non-enzyme-treated cells in binding virus, although overnight incubation of cells with enzyme, while having no effect on influenza virus attachment, reduced EMC virus attachment to 50% of controls (Fig. 1).

The loss of sialoglycopeptides into the supernatant fluid was accompanied, as expected, by a reduction in the size of the sialoglycoproteins associated with the cells. This was shown by analysis of membranes prepared from enzyme-treated and control cells by electrophoresis on 5-6% polyacrylamide gels. Human erythrocyte membranes contain the components PAS-1 to PAS-4 (Burness & Pardoe, 1981; Enegren & Burness, 1977; Steck, 1974). Changes in these components were obvious within minutes of adding the chymotrypsin to cells (compare Fig. 2a
Table 1. Effect of chymotrypsin on HA inhibitory properties of glycophorin

<table>
<thead>
<tr>
<th>Incubation time (min)</th>
<th>EMC virus</th>
<th>Influenza virus</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Enzyme-treated</td>
<td>Control</td>
</tr>
<tr>
<td>15</td>
<td>19</td>
<td>38</td>
</tr>
<tr>
<td>30</td>
<td>59</td>
<td>29</td>
</tr>
<tr>
<td>45</td>
<td>38</td>
<td>59</td>
</tr>
<tr>
<td>60</td>
<td>38</td>
<td>38</td>
</tr>
<tr>
<td>120</td>
<td>38</td>
<td>38</td>
</tr>
<tr>
<td>180</td>
<td>38</td>
<td>77</td>
</tr>
<tr>
<td>240</td>
<td>59</td>
<td>77</td>
</tr>
</tbody>
</table>

and b) and after 4 h incubation the original components were converted almost completely to lower mol. wt. components (result not shown).

Effect of chymotrypsin on glycophorin

Glycophorin preparations were incubated in the presence or absence of chymotrypsin for various lengths of time after which the samples were cooled in ice before measuring their HA inhibitory properties (Table 1). Incubation of glycophorin with enzyme for up to 4 h had no marked effect on its HA inhibitory properties against EMC virus which varied in this experiment from 50 to 200% of controls, a variation not uncommon with an assay like HA inhibition which uses doubling dilutions. Chymotrypsin digestion of glycophorin apparently caused a reduction in its inhibition of influenza virus HA between 40 and 75% of controls in this particular experiment, a change which was marginally significant at the most. The conclusion was, therefore, that chymotrypsin digestion of glycophorin had little effect on its HA inhibitory properties against either virus.

To confirm that chymotrypsin was active against glycophorin, digests were examined by electrophoresis on 5-6% polyacrylamide gels. As described previously (Burness & Pardoe, 1981; Enegren & Burness, 1977; Steck, 1974), undigested glycophorin preparations consisted predominantly of PAS-1 and PAS-2, the dimer and monomer forms of glycophorin A (Furthmayr & Marchesi, 1976; Marton & Garvin, 1973), together with small amounts of PAS-3 and PAS-4 (Fig. 2c). Preparations incubated with chymotrypsin for as little as 15 min, the shortest incubation time examined, produced a markedly different SDS-gel electrophoretic profile compared with undigested material in that PAS-1 and PAS-2 were greatly diminished and a component of mobility between that of PAS-2 and PAS-3 became evident (result not shown). The changes were more pronounced by 1 h by which time PAS-1 and PAS-2 were barely detectable being replaced by three components with greater mobility (Fig 2d). These three components survived incubation with chymotrypsin for more than 18 h, the only change noted being an increase in the amount of the middle component at the expense of the slowest moving component of the three (result not shown). Glycophorin incubated for 18 h in the absence of chymotrypsin had an electrophoretic profile indistinguishable from the original unincubated glycophorin (result not shown).

Separation of glycophorin chymotryptic peptides by gel filtration

Glycophorin was digested with chymotrypsin for 18 h then assayed for HA inhibitory activity and examined by gel filtration. The material eluted in six peaks from Sephadex G-150 using absorbance at 230 nm for detection (Fig. 3), five of the peaks corresponding to peptides CH-1 to CH-5 reported previously (Tomita et al., 1978) based on elution position and, as will be shown below, on amino acid composition. The sixth peak, CH-0, which eluted close to the void volume was not reported previously (Tomita et al., 1978), although a sixth peptide, CH-6, was described which, being insoluble, was removed by centrifugation before gel filtration was attempted both in the studies reported here and in previous studies (Tomita et al., 1978).
Fig. 3. Gel filtration of a chymotryptic digest of glycophorin. $^{3}$H-Acetylated or $^{131}$I-labelled glycophorin was incubated with chymotrypsin for 18 h then examined by gel filtration on Sephadex G-150 in 0.1 M-ammonium acetate pH 6.8. Fractions were analysed for $^{3}$H (---), $^{131}$I (-----) or absorbance at 230 nm (----). CH-0 to CH-5 indicate names assigned to the individual peaks.

Detection of the peptides by u.v. absorption measurements was not a particularly sensitive method for analysis of gel filtration eluates and, therefore, other detection methods were investigated. We have shown previously that glycophorin can be conveniently labelled with $[^{3}$H]acetic anhydride (Pardoe & Burness, 1980). Gel filtration of $^{3}$H-acetylated glycophorin digests revealed that all peptides except CH-5 were radioactive, although CH-3 was of low specific radioactivity (Fig. 3). Peptides CH-3 and CH-5 lack lysine (Fig. 5), the amino acid predominantly acetylated by acetic anhydride at neutral pH, although hydroxylys in serine and, to a slight extent, tyrosine are occasionally attacked (Fraenkel-Conrat, 1962). Despite the low level of radioactivity in CH-3, $^{3}$H-acetylated glycophorin was frequently used in the present studies since, when combined with u.v. absorbance measurements, it facilitated identification of the individual chymotryptic peptides.

More recently we have found that labelling of glycophorin with $^{131}$I gave a product which had very high specific radioactivity ($3 \times 10^7$ ct/min/µg protein). Peptides CH-0 to CH-3 were readily labelled by this method, while peptides CH-4 and CH-5 which lack tyrosine (Fig. 5) were unlabelled (Fig. 3). $^{131}$Iodination therefore promises to be a useful method for future studies since peptides CH-0 to CH-3, unlike CH-4 and CH-5, are glycosylated (Fig. 5) and are thus the peptides of most interest when looking for fragments with receptor-like activity for EMC and influenza viruses.

The fractions comprising each of peaks CH-0 to CH-5 derived from $^{3}$H-labelled glycophorin were combined, lyophilized to remove the ammonium acetate used for gel filtration and the biological, chemical and physical properties of the six components examined further as described next.

**Biological properties of the individual chymotryptic peptides**

To determine which component(s) in chymotryptic digests was responsible for HA inhibition, each of the peptides separated by gel filtration was adjusted to approximately the same protein concentration and tested for biological activity (Table 2). The results were quite clear: of the
Fig. 4. Sucrose density gradient analysis of chymotryptic peptides of $^3$H-labelled glycophorin. Chymotryptic peptides separated by gel filtration (Fig. 3) were analysed on sucrose gradients by centrifugation at 234000 g (a to d) for 6.5 h in the absence or (e to h) for 22.5 h in the presence of 0.1% SDS. Sedimentation is from left to right. Fractions were examined for absorbance at 280 nm to detect the bovine serum albumin marker (○), and for radioactivity (●). P indicates pellet at bottom of centrifuge tube.

Table 2. **HA inhibitory activity of chymotryptic peptides of glycophorin**

<table>
<thead>
<tr>
<th>Sample</th>
<th>EMC virus</th>
<th>Influenza virus</th>
</tr>
</thead>
<tbody>
<tr>
<td>Original glycophorin</td>
<td>20</td>
<td>71</td>
</tr>
<tr>
<td>Chymotryptic digest</td>
<td>15</td>
<td>35</td>
</tr>
<tr>
<td>CH-0</td>
<td>41</td>
<td>285</td>
</tr>
<tr>
<td>CH-1 to CH-5</td>
<td>&gt;1</td>
<td>&gt;2</td>
</tr>
</tbody>
</table>

Chymotryptic peptides, only component CH-0 showed HA inhibition. On a weight for weight basis, CH-0 was more active than the original, undigested or the digested glycophorin. In contrast, peptides CH-1 to CH-5 were completely inactive within the limits of detection of HA inhibition measurements.

**Chemical nature of chymotryptic peptides**

Peptides CH-1, CH-2 and CH-3 were expected to be sialoglycopeptides by comparison with previous results (Tomita et al., 1978). This was confirmed by sialic acid assays which also
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(a) Ser Ser Thr Thr Gly Val Ala Met His Thr Thr Ser Ser Ser Val Ser Lys Ser Tyr
    CH-2
  10  20
Ile Ser Ser Gin Thr Asn Asp Thr His Lys Arg Asp Thr Tyr Ala Ala Thr Pro Arg Ala
    CH-3
  30  40
His Glu Val Ser Glu Ile Ser Val Arg Thr Tyr Pro Pro Glu Glu Thr Gly Glu
  50  60
Arg Val Gin Leu Ala His His Phe Ser Glu Pro Glu Ile Thr Leu Ile Ile Phe Gly Val
    CH-6
  70  80
Met Ala Gly Val Ile Gly Thr Ile Leu Leu Ile Ser Tyr Gly Ile Arg Arg Leu Ile Lys
  90 100
CH-4
Lys Ser Pro Ser Asp Val Lys Pro Leu Pro Ser Pro Asp Thr Asp Val Pro Leu Ser Ser
  110 120
CH-5
Val Glu Ile Glu Asn Pro Glu Thr Ser Asp Gin
  130
(b) CH-1 CH-6 CH-4 CH-5

** ******** ** * * * **

CH-2 CH-3 CH-6 CH-4 CH-5

** ******** ** * * * **

CH-2 CH-0 CH-5

** ******** ** * * * **

Fig. 5. (a) The amino acid sequence, sites of glycosylation and chymotrypsin-sensitive bonds in glycophorin based on published data. (b) Schematic representation of the peptides generated by digestion of glycophorin with chymotrypsin. Peptide CH-1 is produced when the bond between amino acids 34 and 35 remains uncleared (Tomita et al., 1978). CH-0 is thought to be produced by failure to cleave bonds between amino acids 64 and 65 and between 98 and 99. *, Sialo-oligosaccharide side chains; †, bonds cleavable by chymotrypsin.

showed that CH-0 contained sialic acid and that CH-4 and CH-5 contained small amounts, possibly arising from contamination with the early eluting sialoglycopeptides (Table 3).

The amino acid compositions of peptides CH-0, CH-1, CH-2 and CH-3 were determined (Table 3). Under the analytical conditions used, galactosamine which is present in glycophorin (Thomas & Winzler, 1969) co-eluted with valine which therefore could not be quantified. These amino acid analyses were single determinations on column peaks which had not been purified further following chromatography. Under the circumstances, it was satisfying that the amino acid residues calculated for CH-1, CH-2 and CH-3 were in most cases identical to those reported previously (Tomita et al., 1978); they are given in parentheses in Table 3.

There are several differences in the amino acid composition of CH-0 compared with CH-1, CH-2 and CH-3, the most noteworthy difference being the presence of phenylalanine which is absent from the latter three peptides (Table 3).

Physical properties of chymotryptic peptides

Separation of substances by gel filtration is related to their molecular size (Andrews, 1964). Having identified CH-1, CH-2 and CH-3 from their amino acid composition, their mol. wt. can be calculated from their reported chemical composition (Tomita et al., 1978). The elution volume of CH-0 from Sephadex (Fig. 3) compared with those for CH-1, CH-2 and CH-3 was
Table 3. Chemical composition of chymotryptic peptides of glycophorin

<table>
<thead>
<tr>
<th>Peptide</th>
<th>CH-0</th>
<th>CH-1</th>
<th>CH-2</th>
<th>CH-3</th>
<th>CH-4</th>
<th>CH-5</th>
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</thead>
<tbody>
<tr>
<td>Asp*</td>
<td>3.1</td>
<td>3.1</td>
<td>2.8</td>
<td>0.6</td>
<td>0.0</td>
<td>0.0</td>
</tr>
<tr>
<td>Thr</td>
<td>7.3</td>
<td>10.2</td>
<td>7.6</td>
<td>3.3</td>
<td>0.0</td>
<td>0.0</td>
</tr>
<tr>
<td>Ser</td>
<td>8.1</td>
<td>9.7</td>
<td>8.8</td>
<td>2.6</td>
<td>0.0</td>
<td>0.0</td>
</tr>
<tr>
<td>Pro</td>
<td>6.0</td>
<td>3.2</td>
<td>1.2</td>
<td>0.0</td>
<td>0.0</td>
<td>0.0</td>
</tr>
<tr>
<td>Glu</td>
<td>4.9</td>
<td>7.7</td>
<td>2.3</td>
<td>3.0</td>
<td>0.0</td>
<td>0.0</td>
</tr>
<tr>
<td>Gly</td>
<td>6.1</td>
<td>2.0</td>
<td>0.8</td>
<td>0.0</td>
<td>0.0</td>
<td>0.0</td>
</tr>
<tr>
<td>Ala</td>
<td>4.8</td>
<td>4.3</td>
<td>1.2</td>
<td>3.1</td>
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<td>0.0</td>
</tr>
<tr>
<td>Val</td>
<td>1.3</td>
<td>0.9</td>
<td>0.9</td>
<td>0.0</td>
<td>0.0</td>
<td>0.0</td>
</tr>
<tr>
<td>Met</td>
<td>7.1</td>
<td>2.0</td>
<td>1.0</td>
<td>0.0</td>
<td>0.0</td>
<td>0.0</td>
</tr>
<tr>
<td>Ile</td>
<td>1.8</td>
<td>2.4</td>
<td>1.7</td>
<td>0.9</td>
<td>0.0</td>
<td>0.0</td>
</tr>
<tr>
<td>Leu</td>
<td>2.1</td>
<td>2.9</td>
<td>1.9</td>
<td>0.2</td>
<td>0.0</td>
<td>0.0</td>
</tr>
<tr>
<td>Tyr</td>
<td>2.4</td>
<td>2.8</td>
<td>1.9</td>
<td>2.4</td>
<td>0.0</td>
<td>0.0</td>
</tr>
<tr>
<td>Phe</td>
<td>3.2</td>
<td>3.6</td>
<td>0.9</td>
<td>2.4</td>
<td>0.0</td>
<td>0.0</td>
</tr>
<tr>
<td>Sialic acid†</td>
<td>0.30</td>
<td>1.28</td>
<td>1.11</td>
<td>0.57</td>
<td>0.03</td>
<td>0.01</td>
</tr>
</tbody>
</table>

* Amino acid data expressed as relative molar ratios based on phenylalanine as 2 for CH-0, and isoleucine as 2, 1 and 1 respectively for CH-1, CH-2 and CH-3.
† Number in parentheses are expected values, taken from Tomita et al. (1978).
‡ No entry indicates no determination or, in the case of valine, result erroneous due to co-elution with galactosamine.
§ - denotes 0.1 residue or less.
‖ Expressed as mg sialic acid per mg protein.

Table 4. Molecular weights of chymotryptic peptides from electrophoresis on 12.5% polyacrylamide gels

<table>
<thead>
<tr>
<th>Peptide</th>
<th>Mol. wt.</th>
</tr>
</thead>
<tbody>
<tr>
<td>CH-0</td>
<td>30000</td>
</tr>
<tr>
<td>CH-1</td>
<td>22300</td>
</tr>
<tr>
<td>CH-2</td>
<td>14000</td>
</tr>
<tr>
<td>CH-3</td>
<td>7200</td>
</tr>
</tbody>
</table>

* Calculated from published chemical composition (Tomita et al., 1978).

Chymotryptic digests of glycophorin were also examined by electrophoresis on 12.5% polyacrylamide gels containing 0.1% SDS and 8 M-urea (Swank & Munkres, 1971). Undigested glycophorin ran predominantly in the dimer form (i.e. PAS-1) on these gels and penetrated a short distance only (Fig. 2e). Chymotryptic peptides were well separated from each other (Fig. 2f) and from their mobilities estimates were made of their apparent mol. wt. by comparison with the mobilities of mol. wt. markers run on parallel gels. The values obtained for CH-1, CH-2 and CH-3 were in reasonable agreement with mol. wt. calculated from their composition (Table 4). CH-0 had an apparent mol. wt. of 30000 which was less than half that obtained by gel filtration in the absence of detergents.

Tests for biological activity of glycophorin and its chymotryptic peptides were conducted in PBS. Therefore, it was considered necessary to get some idea of the nature of the peptides in physiological solution rather than in the SDS used for electrophoresis or the ammonium acetate used for chromatography. For this purpose, rate zonal ultracentrifugation on sucrose gradients for 6.5 h was used. Under these conditions, 3H-acetylated CH-1, CH-2 and CH-3 behaved as expected for peptides of their size (Table 4) and hardly sedimented into the gradient (Fig. 4b to
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In contrast, CH-0 was apparently aggregated since all of the radioactivity sedimented to the bottom of the tube (Fig. 4a). The sucrose density gradient centrifugations were repeated in the presence of SDS. Under these conditions, peptides CH-1, CH-2 and CH-3 again behaved as small molecules and sedimented as single peaks (although peak CH-3 may have a shoulder) (Fig. 4b to h) and this time CH-0 no longer sedimented in an aggregated form, but also sedimented as a single peak (Fig. 4e).

DISCUSSION

The purpose of the work described in this report was to identify the peptide(s) with receptor-like (HA inhibitory) properties for EMC and influenza viruses in chymotryptic digests of glycophorin. It was found early in the study that the HA inhibitory activity was a property of one peptide, CH-0. Since CH-0 had not been characterized previously, the study was extended to elucidate the structure of CH-0 and to explain why it alone of the chymotryptic peptides had biological activity.

The amino acid sequence and location of the sialo-oligosaccharide side chains in glycophorin are known (Fig. 5a). Also known are the four bonds cleaved by chymotrypsin to give the well-characterized peptides CH-2 to CH-6; sometimes, the bond between CH-2 and CH-3 remains uncleaved giving rise to peptide CH-1 (Figs. 5b). Attempts to work out the structure of CH-0 assumed that cleavage had occurred at no bond other than at one, or more, of these four. Since CH-0 was found to be larger than any of the peptides CH-1 to CH-6, it was considered to be made up of some combination of them.

Characterization of CH-0 was difficult, firstly because the amount found in chymotryptic digests varied, possibly because of the second difficulty which was that CH-0 was not particularly soluble in simple aqueous solvents. Nevertheless, with the availability of extensive information on the structure of glycophorin and its chymotryptic peptides, sufficient information was gathered to suggest that CH-0 had the structure: CH-3-CH-6-CH-4 (Fig. 5b). The evidence for this is as follows.

(i) Evidence that the sequence for CH-0 includes CH-3. CH-0 contained sialic acid (Table 3). Therefore, it must have included within its structure the sialoglycopeptides CH-2 and/or CH-3. The ratio of sialic acid to protein in CH-0 is about 0.3 (Table 3). Since any peptide containing CH-2, which is rich in sialic acid, would have a ratio of 0.67 or higher, clearly CH-2 was not present in CH-0. In fact, the only possible polypeptides with ratios close to 0.3 are CH-3-CH-6-CH-4 (ratio 0.27) and CH-3-CH-6 (ratio 0.35). These two peptides can be distinguished by amino acid analysis as discussed next.

(ii) Evidence that CH-0 contains CH-4. The peptide CH-3-CH-6 would contain neither aspartic acid nor lysine (Fig. 5a). In contrast, both CH-4 (Tomita et al., 1978) and CH-0 both contain three residues of each, indicating that the structure for CH-0 cannot be CH-3-CH-6 but could be CH-3-CH-6-CH-4.

(iii)Evidence that CH-0 contains CH-6. If CH-0 contains CH-3 and CH-4, it must also contain CH-6, which is located between them in glycophorin (Fig. 5b). Direct evidence to support this also came from amino acid analyses. Peptide CH-6 encompasses the only region in glycophorin containing phenylalanine (amino acids 68 and 78) and the same region contains one of the two methionine residues (amino acid 81) in glycophorin. Since CH-0 also contained two phenylalanines and one methionine (Table 3), it is clear that CH-6 was present.

Further evidence that CH-0 has the structure CH-3-CH-6-CH-4 is provided by the similarity in overall amino acid composition to that predicted knowing the individual compositions for CH-3, CH-4 and CH-6 (Table 5). Although the composition found for CH-0 differed slightly from that predicted, this can be expected since the compositions we found for CH-1, CH-2 and CH-3, although close to, were not identical to those published previously (Tomita et al., 1978). The most worrisome differences between the amino acid composition of CH-0 and that predicted for CH-3-CH-6-CH-4 was in the apparent deficiency of proline and glutamic acid in CH-0. However, the value we found for glutamic acid in CH-3 was also much lower than previously reported values (Tomita et al., 1978). Attempts to construct on paper a polypeptide of composition closer to that found for CH-0 by considering cleavages of bonds not normally
Table 5. Comparison of the amino acid composition of CH-0 with that of putative polypeptides

<table>
<thead>
<tr>
<th>Amino acid</th>
<th>CH-3-CH-6</th>
<th>CH-0</th>
<th>CH-3-CH-6-CH-4</th>
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<tr>
<td>Asp</td>
<td>0</td>
<td>3</td>
<td>3</td>
</tr>
<tr>
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<td>7</td>
<td>6</td>
</tr>
<tr>
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<td>8</td>
<td>7</td>
</tr>
<tr>
<td>Pro</td>
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<td>5</td>
<td>5</td>
</tr>
<tr>
<td>Val</td>
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<td>ND†</td>
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<td>1</td>
<td>1</td>
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</tr>
<tr>
<td>Arg</td>
<td>5</td>
<td>3</td>
<td>5</td>
</tr>
</tbody>
</table>

* Compositions of CH-3-CH-6 and CH-3-CH-6-CH-4 were calculated from the composition of the individual peptides (Tomita et al., 1978). The composition for CH-0 is based on data given in Table 3.
† ND, Not determined.

broken by chymotrypsin were unsuccessful. Contamination of CH-0 with other peptides was possibly responsible for the discrepancies in amino acid composition from those predicted. Unfortunately, the hydrophobic nature of CH-0 makes it difficult to purify by conventional techniques available to us and so further characterization is not presently under way.

A question which might be raised is why bonds between CH-3 and CH-6 and between CH-6 and CH-4 were not cleaved in CH-0 but were cleaved in other glycoporin molecules to yield the CH-3, CH-6 and CH-4 seen, for instance, in Fig. 3. One answer is that all glycoporin molecules are not cleaved identically; in some cases, glycoporin is cleaved by chymotrypsin to give CH-1 while in other cases CH-1 is cleaved further to CH-2 and CH-3 (Fig. 5b). Similarly, trypsin treatment of glycoporin in some cases yields a large peptide T-1 while sometimes T-1 is cleaved further (Tomita et al., 1978). Isolated CH-1 and T-1 are refractory to further digestion with chymotrypsin or trypsin respectively, suggesting that certain glycoporin molecules have structural peculiarities, additional glycosylation, for instance, which protects bonds within CH-1, T-1 and perhaps CH-0 from further proteolytic cleavage.

Sialic acid residues in glycoporin are apparently required for HA inhibitory activity since their removal with neuraminidase inactivates glycoporin (Burness & Pardoe, 1981). Therefore, it would be expected that the region of CH-0 containing the sialic acid residues was the biologically active part. Assuming that CH-0 had the structure CH-3-CH-6-CH-4, the CH-3 region of CH-0 would supply the sialic acid moieties. Two other chymotryptic peptides, CH-1 (consisting of CH-2-CH-3) and CH-3 itself, contain common CH-3 structures identical to those found in CH-0 (Fig. 5b) and yet neither had HA inhibitory activity. There are three possible reasons for this.

First, perhaps the CH-3 region in CH-0 did not have exactly the same structure as that found in CH-3 itself, or in CH-1. For instance, CH-0 may have contained additional or modified carbohydrate side chains, and, in fact, such putative differences could have been the reason why bonds within CH-0 resisted further digestion.

A second possibility is that cleavage of CH-1 and CH-3 from glycoporin, or from CH-0 if this is an intermediate, causes a conformational change which results in inactivation. A third possible reason for the HA inhibitory properties of CH-0 is that, unlike CH-1 and CH-3, it forms aggregates in aqueous solvents.

Evidence already exists that aggregation leads to increased capacity to inhibit viral haemagglutination. Several groups showed that orosomucoid polymerized by chemical or physical
methods was a more potent inhibitor of influenza virus than was the non-polymerized glycoprotein (Morawiecki & Lisowska, 1965; Whitehead & Winzler, 1968; Barclay et al., 1969) as was predicted by Fazekas de St. Groth & Gottschalk (1963).

Evidence that CH-0 aggregates is based on a comparison of mol. wt. obtained by various procedures. Assuming CH-0 is CH-3:CH-6:CH-4, its mol. wt. should be about 13000 from its amino acid and sialo-oligosaccharide composition. In fact, CH-0 had an apparent mol. wt. of greater than 64000 to 67000 based on gel filtration (Fig. 3) or sucrose density gradient sedimentation (Fig. 4a) and, even in detergents, a mol. wt. of 30000 was obtained by SDS-polyacrylamide gel electrophoresis (Fig. 2f). In its behaviour, CH-0 was like the parent molecule, glycoporphin which was found to be aggregated when examined on sucrose gradients (Pardoe & Burness, 1981) and which runs predominantly in the dimer form during SDS-gel electrophoresis (Furthmayr & Marchesi, 1976; Marton & Garvin, 1973). Such aggregation which, as in the case of glycoporphin, is due to the presence of the hydrophobic peptide, CH-6 (Furthmayr & Marchesi, 1976), could have caused CH-0 to form multivalent structures which bound to virus sufficiently strongly to withstand the competition by erythrocytes for virus in HA inhibition tests. In contrast, although CH-1 and CH-3 had the same active binding site for virus as CH-0, these peptides remained in the monomeric form (Fig. 4b to d), were easily displaced from virus by erythrocytes and therefore did not inhibit HA. These possible explanations for CH-0 activity are under further investigation.

It was previously predicted, based on the effect of trypsin on human erythrocytes, that one or more of the sialic acid moieties attached to serine at positions 44 and 47 of glycoporphin and threonine at position 50 (i.e. all within the CH-3 region) are involved in the attachment of EMC and influenza viruses to red cells (Enegren & Burness, 1977). It is satisfying that the study reported here leads to conclusions which are consistent with the earlier predictions.

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REFERENCES


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