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Evidence for a single glycan moiety in rabbit serum transferrin and location of the glycan within the polypeptide chain

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The sequential removal of *N*-acetylneuraminic acid from rabbit serum transferrin has been followed by urea-polyacrylamide gel electrophoresis. The electrophoretic pattern is consistent with the presence of a single biantennary glycan chain. From the amino acid sequence of the carbohydrate-containing cyanogen bromide fragment we have shown that the glycan is attached to an asparaginyl side chain at a position equivalent to residue 491 in the sequence of human serum transferrin.

Transferrin; Glycan; Amino acid sequence; Cyanogen bromide fragment; (Rabbit)

1. INTRODUCTION

The transferrins are a class of iron-binding glycoproteins found in serum, egg white, milk and other secretory fluids. Although there is extensive amino acid sequence homology between the different transferrins, the number, position and structure of the glycan moieties vary [1,2]. The glycan chain is always attached to the asparagine residue in the code sequence Asn-X-Ser/Thr, where X can be almost any amino acid but only rarely proline or aspartic acid [3]. In some transferrins this sequence, although present, is not glycosylated, possibly because the potential glycosylation site is sterically inaccessible to oligosaccharide-processing enzymes.

In the case of rabbit serum transferrin there is agreement on the structure of the glycan chain: a biantennary chain with *N*-acetylneuraminic acid residues at the terminal positions [4,5]. There is, however, some uncertainty as to whether the protein bears one biantennary glycan [4,6], and thus two residues of *N*-acetylneuraminic acid, or two biantennary glycans, and therefore four residues of *N*-acetylneuraminic acid, both located within a cyanogen bromide fragment of M_r 24000 [7]. As the X-ray structure of rabbit serum transferrin is currently under investigation [8] it is important to ascertain the number and position of the glycan chains in the protein. We have therefore set out to re-determine the number of residues of *N*-acetylneuraminic acid, and hence the number of glycan chains, on the protein using an electrophoretic technique previously used to demonstrate the presence of a normal amount of *N*-acetylneuraminic acid on a human transferrin variant [9], and also pinpoint the position of the glycan(s) within the polypeptide chain. This study has also extended the amino acid sequence data on rabbit transferrin which at present are limited to the first 12 residues at the amino terminus [6,10] and to those of a carbohydrate-containing pentapeptide [5].

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2. MATERIALS AND METHODS

Transferrin was isolated from iron-saturated rabbit serum by

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ammonium sulphate precipitation followed by ion-exchange chromatography on DEAE-Sepharose.

Neuraminidase (37.5 U), purchased from BDH, was added to 0.5 ml rabbit transferrin (10 mg/ml) in 0.1 M sodium acetate/0.01 M $Ca(NO_3)_2$, pH 5.0, and the solution incubated at 37°C. Samples (40 μl) were removed over a period of 48 h and diluted to 240 μl with 0.2 M $NaHCO_3$/10% (w/v) glycerol. 25 μl of 0.01 M FeNTA was then added and the samples stored at −20°C prior to electrophoresis in the presence of 6 M urea. After 24 h an additional sample (50 μl) was removed, diluted to 100 μl with the original sodium acetate buffer and incubated for 24 h with fresh neuraminidase (7.5 U). As a control transferrin was incubated for 48 h in the absence of neuraminidase. Protein samples were analysed by polyacrylamide gel electrophoresis in the presence of 6 M urea as described [11] except that EDTA was omitted from all buffers and the bisacrylamide concentration was increased to 0.334% (w/v).

Iron-free rabbit transferrin was reduced and carboxamidomethylated as described for human transferrin [12] and digested with cyanogen bromide [13]. After drying in vacuo over NaOH pellets the digest was dissolved in 5% (v/v) formic acid prior to fractionation on a column (1.6 × 90 cm) of Sephacryl S-200 equilibrated with the same buffer. Fractions were examined for the presence of carbohydrate by the orcinol/H_2SO_4 test [14] and analysed by SDS-polyacrylamide gel electrophoresis. Fractions containing the carbohydrate-positive fragment of M_r 24000 were pooled, lyophilised and rechromatographed on the same column to remove small amounts of contaminating fragments.

Amino acid sequencing was carried out using Applied Biosystems 477A pulsed liquid and 470A gas-phase protein sequencers with an on-line 120A PTH-amino acid analyser [15].

3. RESULTS AND DISCUSSION

Fig.1 (slots a–j) shows that when rabbit serum transferrin is incubated with neuraminidase over a period of 48 h there is a two-step reduction in mobility of the iron-saturated protein. No additional decrease in mobility is observed in a sample of transferrin to which has been added fresh neuraminidase after 24 h (fig.1, slot k). In a separate experiment when both rabbit serum transferrin and human serum transferrin were incubated with neuraminidase over a period of 24 h there was once again a two-step decrease in mobility of rabbit transferrin whereas a four-step decrease in mobility was observed with human transferrin. These results are consistent with the sequential removal of two residues of *N*-acetylneuraminic acid from rabbit transferrin. This procedure has already been used to demonstrate the presence of 4 *N*-acetylneuraminic acid residues in the terminal positions of the 2 biantennary glycans on normal human serum

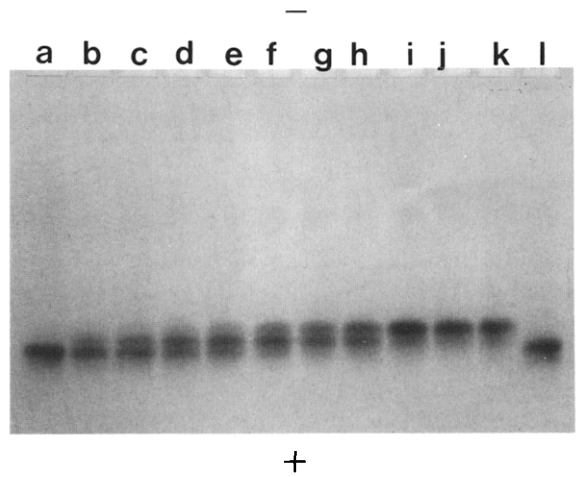

Fig.1. Urea-polyacrylamide gel electrophoresis of diferric rabbit transferrin incubated with neuraminidase. (a) Diferric transferrin. (b–j) Samples taken from reaction mixture at (b) 10 min, (c) 20 min, (d) 40 min, (e) 1 h, (f) 2 h, (g) 3 h, (h) 4 h, (i) 24 h, (j) 48 h. (k) Transferrin incubated for 24 h and a further 24 h with fresh neuraminidase. (l) Transferrin incubated for 48 h without neuraminidase.

transferrin and a variant with abnormal iron-binding properties [9].

From the results with rabbit serum transferrin we can conclude that the protein carries a single biantennary glycan, with two residues of *N*-acetylneuraminic acid at the terminal positions, in agreement with two earlier reports [4,6] rather than two biantennary glycans, with 4 residues of *N*-acetylneuraminic acid at the terminal positions [7]. The figure of two glycan chains/molecule [7] was calculated from the carbohydrate composition of a rabbit serum transferrin preparation [16] possibly contaminated by haemopexin, a carbohydrate-rich protein notoriously difficult to remove from serum transferrins.

All the carbohydrate on rabbit serum transferrin has been shown to be located in a cyanogen bromide fragment of M_r 24000 [7]. Upon tryptic digestion of this fragment a glycopeptide with the sequence Asn(CHO)-Ser-Ser-Leu-Cys was isolated [5]. In order to determine the position of this pentapeptide within the protein we have purified the carbohydrate-containing cyanogen bromide fragment from digests, by gel filtration, and subjected it to automatic amino acid sequencing. The amino acid sequence of the first 63 residues is shown in fig.2. At position 27 no amino acid was detected.

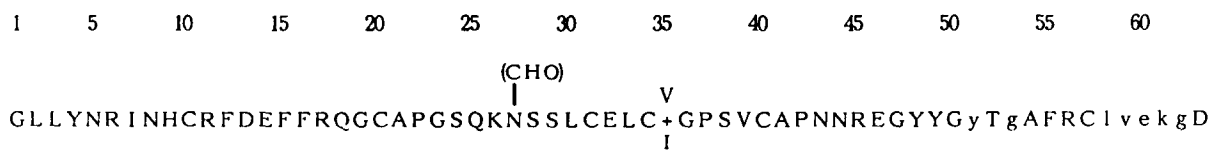

Fig.2. Sequence of carbohydrate-containing cyanogen bromide fragment of rabbit transferrin.

The sequence in the fragment from residue 28 to 31 corresponds to the sequence which follows the glycosylated asparagine residue in the tryptic pentapeptide. The absence of a detectable amino acid at position 27 in the fragment is consistent with the presence of a glycosylated asparagine residue at this position. The tryptic pentapeptide therefore corresponds to residues 27–31 in the fragment.

As the complete amino acid sequences of the homologous proteins human serum transferrin [17], hen ovotransferrin [18] and human lactoferrin [19] are known it is possible to align the partial sequence of the cyanogen bromide fragment from rabbit transferrin with that of the three other transferrins. We can see from fig.3 that the amino-terminal residue in the cyanogen bromide fragment from rabbit transferrin corresponds to residue 465 in the human transferrin sequence, which follows a conserved methionine residue, and the glycosylation site in the rabbit protein is the result of a substitution of an aspartic acid residue at position 491 by an asparagine residue. This is also thought to be the position of the glycosylation site in bovine serum transferrin [19]. The X-ray crystallographic structure of rabbit serum transferrin at a resolution of 3.3 Å has recently been reported [8] and electron density in the region of residue 491 has been interpreted in terms of a glycan chain which appears to interact, via its terminal sialic acid, with a region within the N-terminal lobe.

Although we have only tentatively assigned a tyrosine to position 51 in the carbohydrate-containing fragment from rabbit transferrin (fig.2), a tyrosine residue is present at the corresponding position in human serum transferrin, hen ovotransferrin and human lactoferrin (fig.3). This particular tyrosine is of particular importance as X-ray structural studies [8,20] have shown that it interacts with the iron atom in the C-terminal metal-binding site.

Two hydrophobic amino acids, valine and isoleucine, were found at position 35 in the cyanogen bromide fragment from rabbit transferrin. As the transferrin was isolated from pooled rabbit serum there is the possibility of polymorphism at this position, as has been observed at a number of positions within the amino acid sequence of hen ovotransferrin [21]. The alignment of the region immediately following the valine/isoleucine with the sequences of human serum transferrin, human lactoferrin and hen ovotransferrin (fig.2) was based on that predicted for the corresponding N-terminal region using a multiple alignment algorithm [22] and supported by X-ray structural studies [8,20].

In this study we have shown that only one glycan chain is present on rabbit serum transferrin and located its position within the polypeptide chain. At the same time these studies on the carbohydrate-containing cyanogen bromide fragment have extended the rather limited amino acid

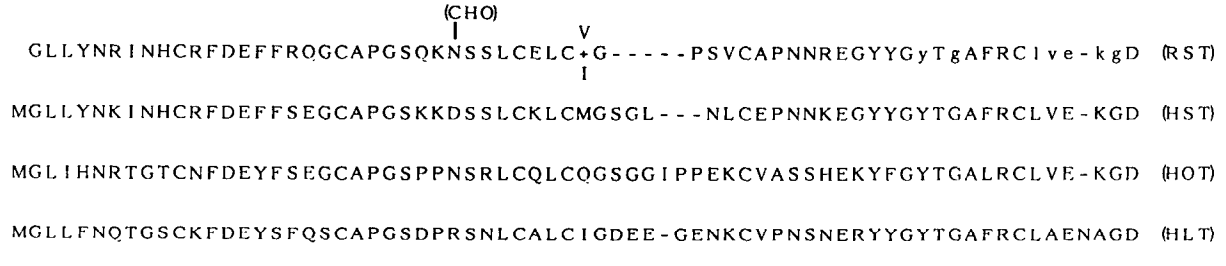

Fig.3. Sequence of cyanogen bromide fragment of rabbit transferrin (RST) aligned with human serum transferrin (HST), hen ovotransferrin (HOT) and human lactoferrin (HLT). Gaps (–) have been inserted to maximise the homology.

sequence data on the protein. Such information is required for correct interpretation of the results of crystallographic studies on rabbit transferrin [8]. Further studies are now in progress to obtain additional sequence data from the other cyanogen bromide fragments of rabbit serum transferrin.

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REFERENCES

[1] Metz-Boutique, M.-H., Jolles, J., Mazurier, J., Schoentgen, F., Legrand, D., Spik, G., Montreuil, J. and Jolles, P. (1984) Eur. J. Biochem. 145, 659–676.
[2] Spik, G., Coddeville, B., Legrand, D., Mazurier, J., Leger, D., Goavec, M. and Montreuil, J. (1985) in: Proteins of Iron Storage and Transport (Spik, G. et al. eds) pp.47–51, Elsevier, Amsterdam, New York.
[3] Kornfeld, R. and Kornfeld, S. (1985) Annu. Rev. Biochem. 54, 631–664.
[4] Leger, D., Tordera, V., Spik, G., Dorland, L., Haverkamp, J. and Vliegenthart, J.F.G. (1978) FEBS Lett. 93, 255–260.
[5] Strickland, D.K., Hamilton, J.W. and Hudson, B.G. (1979) Biochemistry 18, 2549–2554.
[6] Heaphy, S. and Williams, J. (1982) Biochem. J. 205, 611–617.
[7] Strickland, D.K. and Hudson, B.G. (1978) Biochemistry 16, 3411–3418.
[8] Bailey, S., Evans, R.W., Garratt, R.C., Gorinsky, B., Hasnain, S., Horsburgh, C., Jhoti, H., Lindley, P.F., Mydin, A., Sarra, R. and Watson, J.L. (1988) Biochemistry, in press.
[9] Evans, R.W., Williams, J. and Moreton, K. (1982) Biochem. J. 201, 19–26.
[10] Sarra, R. and Lindley, P.F. (1986) J. Mol. Biol. 188, 188–189.
[11] Williams, J., Evans, R.W. and Moreton, K. (1978) Biochem. J. 173, 535–542.
[12] Sutton, M.R. and Brew, K. (1974) Biochem. J. 139, 163–168.
[13] Findlay, J.B.C. and Brew, K. (1972) Eur. J. Biochem. 127, 65–86.
[14] Winzler, R.J. (1954) Methods Biochem. Anal. 2, 279–311.
[15] Bradford, A.P., Howell, S., Aitken, A., James, L.A. and Yeaman, S.J. (1987) Biochem. J. 245, 919–922.
[16] Hudson, B.G., Ohno, M., Brockway, W.J. and Castellino, F.J. (1973) Biochemistry 12, 1047–1053.
[17] Yang, F., Lum, J.B., McGill, J.R., Moore, C.M., Naylor, S.L., Van Bragt, P.H., Baldwin, W.D. and Bowman, B.H. (1984) Proc. Natl. Acad. Sci. USA 81, 2752–2756.
[18] Jeltsch, J.-M. and Chambon, P. (1982) Eur. J. Biochem. 122, 291–295.
[19] Maeda, K., McKenzie, H.A. and Shaw, D.C. (1984) Anim. Blood Grps. Biochem. Genet. 15, 313–322.
[20] Anderson, B.F., Baker, H.M., Dodson, E.J., Norris, G.E., Rumball, S.V., Waters, J.M. and Baker, E.N. (1987) Proc. Natl. Acad. Sci. USA 84, 1768–1774.
[21] Williams, J., Elleman, T.C., Kingston, I.B., Wilkins, A.G. and Kuhn, K.A. (1982) Eur. J. Biochem. 122, 297–303.
[22] Barton, G. and Sternberg, M.J.E. (1987) J. Mol. Biol. 198, 327–337.