Structural Studies on Individual Components of Bovine Transferrin

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The single-banding components of bovine transferrin from animals homozygous for the four transferrin variants found in the U.K. were isolated. Sedimentation equilibrium ultracentrifugation and sodium dodecyl sulphate–polyacrylamide-gel electrophoresis showed that the bands of a single variant have molecular weights of 77 500 and 73 300 respectively. The different bands of a single variant and single bands of different variants show no evidence of size heterogeneity or of low-molecular-weight peptides being split off after reduction in 6M-guanidine hydrochloride. The two slower bands of a single variant, which both contain 2 molecules of sialic acid/molecule of protein, have the same molecular weight and amino acid composition, and give identical peptide ‘maps’, although differences in composition and peptide ‘maps’ occur between the different variants. The results support the concept that bovine transferrin is essentially a single polypeptide chain, but they do not explain differences in electrophoretic mobility between bands of the same variant which are not produced by differing sialic acid content.

It has been shown previously that transferrin in cattle, when examined by starch-gel electrophoresis, exhibits a complex heterogeneity. Genetic variation is found, but even transferrin from a homozygous individual exhibits multiple bands partly owing to differing numbers of sialic acid residues (Stratil & Spooner, 1970, 1971). Stratil & Spooner (1971) also showed that the two bands of a single variant, each possessing two residues of sialic acid, had the same isoelectric point but showed an increasing difference in charge as the pH was raised above the isoelectric point. To study this remaining heterogeneity further it was therefore decided to determine the amino acid composition and sedimentation behaviour of individual bands from homozygous transferrin variants, and to search for possible differences in the peptide ‘maps’ of these individual components. A preliminary account was presented at the 13th European Conference on Animal Blood Groups and Biochemical Polymorphism (Spooner *et al.*, 1972).

Although most authors have reported that mammalian transferrins have a single-chain structure, it has been suggested by Jepson (1967) for man, and by Efremov *et al.* (1971) for cattle, that transferrin is composed of two subunits. We have looked for such subunits, using sedimentation equilibrium and electrophoresis in polyacrylamide gels in sodium dodecyl sulphate.

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The degree of difference between genetic variants was examined by comparing their peptide ‘maps’ and amino acid compositions.

Materials and Methods

Materials

Transferrin. This was isolated as previously described (Stratil & Spooner, 1971). Briefly, this involved precipitation with rivanol (6,9-diamino-2-ethoxyacridine lactate) obtained from Sigma Chemical Co. Ltd., London, U.K., and then with $(\text{NH}_4)_2\text{SO}_4$, followed by gradient elution from DEAE-Sephadex as described by Stratil & Spooner (1971). The protein appeared homogeneous by sedimentation velocity, electrophoresis on starch gel and immunoelectrophoresis. The designation of transferrin bands 2a, 2b etc. refers to the nomenclature proposed by Stratil & Spooner (1971). Band 2a is the slower electrophoretic band and band 2b is the faster, each with two residues of sialic acid/molecule of protein.

Guanidine carbonate. This was obtained from Hopkin and Williams Ltd., Romford, Essex, U.K., converted into the hydrochloride by dissolving in conc. HCl and used without further purification after adjustment to pH 8.0 with Tris.

Trypsin. This was obtained from Worthington Biochemical Corp., Freehold, N.J., U.S.A.

Dithiothreitol. This was obtained from Calbiochem Ltd., London, U.K.

All other reagents were the best commercially available, and were used without further purification.

Methods

Ultracentrifuge studies. These were carried out on a Beckman model E analytical ultracentrifuge.

Sedimentation-equilibrium studies were performed by using the short-column, high-speed method of Yphantis (1964). Standard double-sector cells, equipped with sapphire windows, 4 mm solution column lengths, and a constant temperature of 25°C were used throughout.

Sedimentation-velocity studies were performed by using schlieren optics in single-sector centrepieces, at constant temperatures between 20° and 21°C, and rotor speeds of 60000 rev./min.

Sodium dodecyl sulphate-polyacrylamide-gel electrophoresis. Polyacrylamide-gel electrophoresis with sodium dodecyl sulphate was performed by the technique of Summers *et al.* (1965). Purified proteins were dissolved in 0.01 M-sodium phosphate buffer, pH 8.0, containing 8 M-urea, and reduced with 20 mM-dithiothreitol for 1 h at room temperature. The mixture was then made 120 mM with respect to iodoacetamide. A portion (5 µg) of protein was then electrophoresed on 8 cm long 4.25% (w/v) gels at a constant current of 10 mA/gel for 3 h at room temperature. The migration distance of each protein was then measured and plotted on a standard curve of similarly treated proteins of known molecular weights.

Starch-gel electrophoresis. This was performed as described previously by Spooner & Baxter (1969) with 0.016 M-Tris-cacodylic acid, pH 7.3, and Connaught hydrolysed starch (batch 262-1; Connaught Medical Research Laboratories, Toronto, Canada).

Immunoelectrophoresis. Rabbit antisera to whole bovine serum and crude transferrin preparations were used to check the purity of fractions by a modification of the micro-immunoelectrophoretic method of Scheidegger (1955). Immunoelectrophoretic patterns demonstrating the purity of some of the fractions used in this investigation are shown by Stratil & Spooner (1971).

Preparation of carboxymethylated chains. The method used was similar to that of Beale & Feinstein (1969). A sample of a 1% (w/v) solution of transferrin in 6 M-guanidine-HCl, pH 8.0, was flushed with N₂ and then made 20 mM with respect to dithiothreitol. After 1 h the reaction mixture was made 60 mM in iodoacetic acid. After a further 1 h each sample was dialysed against 0.15 M-NaCl and then against 0.1 M-NH₄HCO₃, pH 8.5.

Amino acid analysis of chains. Samples (1 mg) of carboxymethylated chains were hydrolysed in 0.5 ml of constant-boiling, glass-distilled HCl for 24 h *in vacuo* at 105°C. Hydrolysates were evaporated to dryness over NaOH pellets in a vacuum desiccator

and then dissolved in 0.067 M-sodium citrate buffer, pH 2.0, ready for amino acid analysis. Samples (approx. 2 and 10 nm) were analysed on a Technicon microanalyser as previously described (Beale & Kent, 1968).

'Fingerprint' studies. Samples (10 mg) of carboxymethylated chains in 1 ml of 0.1 M-NH₄HCO₃, pH 8.5, were digested at 37°C for 4 h by the addition of trypsin to give a protein/enzyme ratio of 50:1 (w/w). 'Mapping' of the soluble tryptic peptides from carboxymethylated chains was done by high-voltage paper electrophoresis at pH 3.5 (in water-acetic acid-pyridine, 89:10:1, by vol.), 3000 V for 1 h at room temperature being applied, and then ascending chromatography in the second dimension in butan-1-ol-water-pyridine-acetic acid (15:12:10:3, by vol.). Peptides were located by dipping the paper in 0.2% (w/v) ninhydrin in acetone and heating at 80°C for 20 min.

Results

Purity

Preparations of homozygous transferrins A, D₁, D₂ and E were first examined for purity by using starch-gel electrophoresis and immunoelectrophoresis. The two main bands, 2a and 2b, were obtained for each type. In Plate 1(a) are shown the 2a preparations from the four transferrin variants on starch-gel electrophoresis and an unfractionated transferrin A preparation. In Plate 1(b) are shown the 2b bands from transferrins A and E, with unfractionated transferrins A and E for comparison.

These purified preparations were then examined by several analytical techniques.

Sedimentation-velocity and electrophoretic examination

Ultracentrifugation. Sedimentation velocities were measured for an unresolved mixture of bands 2a and 2b of transferrin A in 0.017 M-Na₂HPO₄, buffered to pH 7.6 with 0.017 M-KH₂PO₄ and containing 0.15 M-NaCl (*I* = 0.2). The effect of protein concentration is linear and $s_{20,w}^0$ obtained from the intercept was 5.18 S (Fig. 1). Values measured by using single concentrations of single-banding components of transferrins D₁, band 2a, D₂, band 2a, E, band 2a and A, band 2b fell approximately on the same line. The mixture of bands 2a and 2b of transferrin A gave a single symmetrical peak showing no evidence of size heterogeneity.

Sedimentation equilibrium. After the sedimentation-velocity measurements the purified preparations were examined by the meniscus-depletion method of Yphantis (1964). The proteins were examined firstly in 0.017 M-Na₂HPO₄, buffered to pH 7.6 with 0.017 M-KH₂PO₄ and containing 0.15 M-NaCl (*I* = 0.2), and

EXPLANATION OF PLATE I

Starch-gel electrophoresis of the isolated bands of bovine transferrin

(a) The slowest band is shown with two residues of sialic acid (2a) for the four genetic variants of transferrin E, D₂, D₁ and A. Unfractionated transferrin A is shown for comparison. (b) Unfractionated transferrins A and E; in the two central positions the faster bands are shown of these two variants possessing two residues of sialic acid (transferrin A band 2b, and transferrin E band 2b).

EXPLANATION OF PLATE 2

'Fingerprints' of tryptic peptides of carboxymethylated transferrin

These were obtained by electrophoresis at pH 3.5 in the first dimension and by ascending chromatography in butan-1-ol–water–pyridine–acetic acid in the second dimension, developed with ninhydrin. The transferrins run were: (a) A band 2a; (b) A band 2b; (c) A band 2a; (d) E band 2a. (a) and (b) were in one run, (c) and (d) were in another.

Fig. 1. Sedimentation velocity of bovine transferrin preparations

Preparations were analysed in 0.017M- Na_2HPO_4 , buffered to pH 7.6 with 0.017M- KH_2PO_4 and containing 0.15M-NaCl ($I = 0.2$). For details see the text. Transferrins: $\circ$, A band 2a; $\square$, A band 2b; Δ , D₁ band 2a; ∇ , E band 2a; $\bullet$, D₂ band 2a.

then fully reduced and carboxymethylated in guanidine hydrochloride. The results for a typical plot are shown in Fig. 2 for a mixture of components 2a and 2b from transferrin A. By using the value of 0.725 ml/g for the partial specific volume, $\bar{v}$ (Oncley *et al.*, 1947), the molecular weight was calculated to be 77 500, comparable with the value for human transferrin of 76 600 (Mann *et al.*, 1970) measured by the same method. The results for both the aqueous runs and those in 6M-guanidine hydrochloride are shown in Table 1, with those in 6M-guanidine hydrochloride showing a lower molecular weight than those in the aqueous buffer. There is close agreement between the several measurements in one solvent, but the molecular weights in 6M-guanidine hydrochloride are uniformly lower than those in aqueous media, and if the value of $\bar{v}$ is decreased to 0.715 to take account of possible preferential binding of guanidine to the protein (Hade & Tanford, 1967) then this difference is amplified. The differences here are considerably greater than those described by Mann *et al.* (1970), who, using the same value for $\bar{v}$ for both solvents, found little difference in molecular weights.

Sodium dodecyl sulphate-polyacrylamide-gel electrophoresis. The molecular weight of reduced and carboxymethylated transferrin was 73 300 on the sodium dodecyl sulphate-polyacrylamide gels, with bovine serum albumin and immunoglobulin G light chains as standards. There was no evidence for the presence of a smaller component even when gels were loaded with 200 μg of transferrin.

Chemical analysis

Amino acid composition. It has been assumed that the carbohydrate content of bovine transferrin is 6.2%, i.e. the same as that found for human trans-

Fig. 2. Plot of $\log$ (fringe displacement) against square of distance from centre of rotation, r^2

Sedimentation-equilibrium data were obtained at 25°C. (a) Reduced carboxymethylated mixture of bands 2a and 2b of transferrin A (0.07%, w/v, in 6.05M-guanidine hydrochloride-0.04M-Tris-0.05M-2-mercaptoethanol-0.1M-iodoacetamide, pH 8.0) after 64 h at 20 000 rev./min; (b) Mixture of bands 2a and 2b of transferrin A (0.07%, w/v, in phosphate-NaCl buffer, pH 7.6, $I = 0.2$) after 35 h at 16 000 rev./min.

ferrin by Jamieson (1965), and that the tryptophan content is 9 residues/molecule (Mann *et al.*, 1970). Therefore the molecular weight of the peptide chain of the intact transferrin has been taken as 71 000 and the amino acid compositions have been calculated on this weight. The results for 24 h hydrolysates of the four common transferrin variants are shown in Table 2. With transferrins A and E both bands 2a and 2b were studied. There is close agreement between the two bands from a single variant and there are

Table 1. *Molecular weights of bovine serum transferrin*

These were obtained by sedimentation equilibrium at 25°C of an unresolved mixture of bands 2a and 2b of transferrin A. A protein concentration of 0.07% (w/v) was used in the buffer shown. For further details see the Methods section.

	Rotor speed (rev./min)	Mol.wt.	
		$\bar{v} = 0.725$ ml/g	$\bar{v} = 0.715$ ml/g
In phosphate-NaCl, pH 7.6.	16000	77200	
	18000	77800	
	Average	77500	
In 6M-guanidine-HCl, pH 8.0	20000	67900	63600
Reduced carboxymethylated protein	24000	64900	60800
Average		66400	62200

Table 2. *Amino acid compositions of bovine serum transferrin*

Results are not corrected for zero time of hydrolysis; numbers of residues are based on a molecular weight of 77500, less 6.2% carbohydrate, and assuming the presence of 9 residues of tryptophan/molecule.

		Composition (residues/molecule)						
Transferrin	...	E		D ₂	D ₁	A		A
		2a	2b	2a	2a	2a	2b	(mainly 2b) (Efremov <i>et al.</i> , 1971)
Cys*	Band ...	34	33	33	35	34	33	
Asp		81	83	79	80	87	85	87
Thr		32	31	32	35	33	34	31
Ser		38	39	38	41	39	42	32
Glu		59	61	61	63	60	59	56
Pro		28	31	27	28	28	31	32
Gly		44	49	45	50	48	46	49
Ala		49	51	49	54	52	51	55
Val		35	35	34	32	31	32	44
Met		10	9	9	10	10	10	10
Ile		21	21	19	19	20	19	22
Leu		47	44	46	47	44	48	53
Tyr		20	20	21	23	21	22	23
Phe		27	27	26	29	31	30	34
Lys		56	56	55	54	51	50	56
His		17	15	14	16	15	17	16
Arg		23	22	21	22	21	22	24
Total residues		621	627	609	638	625	631	

* As carboxymethyl-Cys.

slightly greater differences between the different variants. The results shown here are similar to those described for human transferrin (Bezborovainy *et al.*, 1968; Mann *et al.*, 1970) and for bovine transferrin A (Efremov *et al.*, 1971). The most striking difference appears to be an appreciably higher leucine content in human transferrin, and higher valine and lower serine contents in the results of Efremov *et al.* (1971) for bovine transferrin.

Peptide analysis. The numbers of lysine and arginine residues found here in bovine transferrin varies from 72 to 79, depending on the particular genetic variant. If there is no duplication of sequences in the molecule then tryptic degradation should produce a maximum of 73–80 peptides. The results for typical tryptic digests of carboxymethylated transferrins, which were found reproducible, are shown in Plate 2. In Plate 2(a) and 2(b) are shown runs carried

Table 3. *Demonstration of the presence (+) or absence (–) of the three tryptic peptides shown in Plate 2 for the four genetic variants studied*

The 'fingerprints' of the soluble tryptic peptides of carboxymethylated transferrin were obtained by electrophoresis at pH 3.5 in one dimension and by ascending chromatography in the second dimension. The 'fingerprints' were then developed with ninhydrin (see the Methods section).

Genetic type	Tp I	Tp II	TpIII
A	+	+	–
D ₁	+	+	–
D ₂	–	+	+
E	+	–	–

out at the same time for bands 2a and 2b of transferrin A. The two peptide 'maps' are essentially identical and at least 65 of the expected peptides can be differentiated. This result does not support the suggestion of Mann *et al.* (1970), who found fewer peptides in 'maps' of human transferrin than expected, that there might be repeating sequences within a single peptide chain as a consequence of a gene duplication. Similarly, 'maps' carried out for bands 2a and 2b of transferrin E were found to be identical. In Plate 2(c) and 2(d) are shown runs in which transferrins A band 2b, and E band 2b, are compared. The peptides TpI and TpII are shown, and the position of peptide TpIII (absent in the illustrated 'maps') is indicated. Table 3 shows which of these peptides are present in the four variants tested. Transferrins A, D₂ and E differ in one or more peptides but transferrin D₁ is indistinguishable from transferrin A.

Discussion

Our finding of 77 500 for the molecular weight of bovine transferrin agrees with our previous estimate from Sephadex G-200 chromatography of 74 000 (Stratil & Spooner, 1971). It also agrees with that determined for human transferrin by Mann *et al.* (1970) (76 600 by using sedimentation equilibrium) and that by Palmour & Sutton (1971) for human, rabbit and frog transferrins (approx. 76 000 by using sedimentation equilibrium, sodium dodecyl sulphate–polyacrylamide-gel electrophoresis and gel filtration). However, Efremov *et al.* (1971) reported rather lower molecular weights of 67 000 (from sedimentation and viscosity data) and 72 400 (by sedimentation and diffusion) for bovine transferrins.

Although the molecular weight (66 400) measured after reduction in guanidine hydrochloride was rather lower than that found in aqueous buffer, it was far too high to suggest dissociation into half-molecules of the type proposed by Jepson (1967) or by Efremov *et al.* (1971).

The single bands seen in sodium dodecyl sulphate–polyacrylamide gels after full reduction, with an estimated molecular weight of 73 300, confirm that the bovine transferrin molecule is essentially a single polypeptide chain. This is in agreement with the conclusions of many other workers studying the transferrins of several species [e.g. human (Bezkorovainy & Grohlich, 1967; Mann *et al.*, 1970); chicken, human and rabbit (Greene & Feeney, 1968); human, rabbit and frog (Palmour & Sutton, 1971)]. Moreover there was no evidence in our polyacrylamide-gel studies for the separation of any lower-molecular-weight peptide that might account for the slight fall in molecular weight seen in guanidine hydrochloride.

The sedimentation–equilibrium and sedimentation-velocity studies failed to detect any difference in size between the individual bands of transferrin A or between these bands and those of other genetic variants. Although these centrifuge studies could not be expected to detect small size differences that might be related to the banding of a single variant, they are in accord with the failure to detect differences in the 'maps' of soluble tryptic peptides.

Although we find small differences in amino acid composition between the individual genetic variants in cattle, there are no significant differences between the individual bands of a single genetic variant. Moreover, the bands of a single transferrin variant give identical peptide 'maps'. Three of the genetic variants, transferrins D₂, E and A, have distinct peptide 'maps', whereas that of transferrin D₁ appears identical with that of transferrin A. The studies on amino acid composition also indicate that there are detectable differences between the different variants. In all of the preparations studied there are lower valine contents than those reported by Efremov *et al.* (1971) and there is much less difference between the different genetic variants reported here than between the different preparations of transferrin A as shown by Efremov *et al.* (1971).

The results in the present paper show that the two bands of a single genetic variant both possessing 2 residues of sialic acid/molecule of protein do not differ in molecular weight, amino acid composition or peptide 'maps'. They are clearly controlled by a common gene, but the difference giving rise to the banding is still unclear. Further studies on related species may suggest the stage at which a possible mutation giving rise to pair formation took place.

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