Single-Chain Nature of Human Serum Transferrin*

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ABSTRACT: Human serum transferrin contains two identical iron binding sites, and two identical carbohydrate groups per molecule. These facts imply a two-subunit structure for the protein, and, in fact, support for such a structure has appeared in the literature, though there are also contradictory reports to indicate that the molecule contains only a single polypeptide chain. We have examined this problem using the techniques of sedimentation equilibrium, intrinsic viscosity measurement, and gel filtration in 6 M guanidine hydrochloride. The results of these studies indicate that the observed native molecular weight of human serum transferrin (77,000) is not altered upon reduction, reduction and carboxymethylation, or oxidation and complete denaturation in 6 M guanidine hydrochloride. None of these data suggest anything other than a single continuous polypeptide chain in human serum transferrin. Peptide mapping experiments lead to the observation of only about two-thirds of the expected number of peptides from tryptic hydrolysis of human serum transferrin. This result, together with the previously reported presence of duplicate binding sites and carbohydrate moieties, raises the possibility that some portions of the polypeptide chain may occur in duplicate.

Human serum transferrin[1] is a non-heme iron binding protein which is believed to play a role in the transfer of iron from storage areas to the erythroblasts (Fletcher and Huehns, 1968). The protein has been extensively studied, and numerous reports of the native molecular weight have appeared in the literature. These values range from a high value of 93,000 (Bezkorovainy and Grolich, 1967) to a low value of 68,000 (Charlwood, 1963).

Transferrin possesses two equivalent iron binding sites (Aisen *et al.*, 1966) and two identical carbohydrate side chains (Jamieson, 1965) per molecule. These experimental findings suggest that this protein may be composed of two subunits. Indeed one worker (Jeppson, 1967) has reported that the molecule is composed of two similar polypeptide chains. However data from other laboratories (Bezkorovainy and Grolich, 1967; Greene and Feeney, 1968) suggest that the native molecule is composed of a single polypeptide chain.

We have undertaken a study of the subunit nature of transferrin with three possibilities in mind. First, that transferrin may be a single polypeptide chain. Second, that the molecule may be composed of two subunits, the interactions between which are susceptible to disruption under the conditions commonly employed in subunit studies (6 M Gu·HCl,[2] 2-mercaptoethanol). Third, that the molecule may possess two or more polypeptide chains which are interconnected by some unusual cross-link, resistant to cleavage by ordinary chemical means.

These possibilities have been evaluated by the techniques of sedimentation equilibrium, intrinsic viscosity of the random coil, gel filtration in 6 M Gu·HCl, tryptic fingerprinting, and quantitative amino-terminal acid analyses. The results obtained from these studies can only be interpreted in terms of a single polypeptide chain structure for transferrin.

Methods

Materials. The transferrin used in this study was a generous gift of Dr. Philip Aisen. The protein was judged homogeneous by sedimentation velocity and by electrophoresis on starch gel, pH 8.9, and on disc gel, pH 3.5.

Agarose (6%) was obtained from Bio-Rad Laboratories (A-5M) and 4% agarose was obtained from Pharmacia Fine Chemicals, Inc. (Sepharose 4B).

Gu·HCl was obtained from Baker and purified by the method of Tanford and Nozaki (1967) or purchased from Heico, Inc. (ultra-high purity), and used without further purification. Iodoacetic acid was obtained from Matheson, Coleman & Bell and recrystallized from hexane. TPCK trypsin was obtained from Worthington. All other reagents were the best commercially available, and used without further purification.

Ultracentrifuge Studies. Sedimentation equilibrium studies were performed using the short-column, high-speed technique of Yphantis (1964).

Samples for study were dissolved in the appropriate solvent and dialyzed for at least 24 hr prior to ultracentrifugal analysis. In studies designed to promote dissociation to the ultimate subunit, transferrin was reduced in 6 M Gu·HCl–0.1 M 2-mercaptoethanol (pH 8.6) prior to dialysis. In cases where carboxymethylation was desired, the reduced protein was carboxymethylated with a slight excess of iodoacetic acid at pH 8.6. Sedimentation equilibrium studies were performed at constant temperatures between 20 and 25° using 3-mm solution columns in double-sector cells equipped with sapphire windows.

The attainment of equilibrium was determined experimentally by measuring fringe displacements at given radial posi-

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[1] We shall refer to human serum transferrin as transferrin or apotransferrin (the iron-free protein) throughout the remainder of the text. Transferrins from other sources will be identified as such.

[2] Abbreviations used are: guanidine hydrochloride, Gu·HCl; L-(1-tosylamido-2 phenyl)ethyl chloroethyl ketone, TPCK.

tions on successive photographs. A water blank was run after each experiment in order to correct for window distortion.

All plots of sedimentation equilibrium data were linear, indicating homogeneity within experimental error. Molecular weights were calculated using the expression

$$M = \frac{2RT\dfrac{\mathrm{d}\ln c}{\mathrm{d}r^2}}{\omega^2(1 - \phi'\rho)}$$

in which ϕ' is the effective partial specific volume of the protein, ω is the rotor angular velocity, and ρ is the solvent density. The values for d ln c/dr^2 were obtained from least-squares analysis of plots of log fringe displacement *vs.* the square of the radial distance for points above 100-μ fringe displacement.

The partial specific volume, $\bar{v}$, for transferrin has been determined by Oncley *et al.* (1947) as 0.725 ml/g. This value of $\bar{v}$ has been used for ϕ' in the calculation of molecular weights of the native protein in dilute salt solutions. However, the choice of ϕ' in concentrated Gu·HCl solutions is somewhat ambiguous. The effective partial specific volume, ϕ', is an expression which collects the partial specific volume, $\bar{v}$, which can be measured pycnometrically, and additional terms in the expression for sedimentation equilibrium which arise from preferential solvent interactions. In dilute salt solutions, preferential solvent interactions are minimal, and ϕ' is essentially equal to $\bar{v}$. Hade and Tanford (1967) have shown from isopiestic studies, that in concentrated Gu·HCl solutions, ϕ' may be decreased by as much as 0.01 ml/g owing to preferential guanidination of protein. We have considered two possibilities in calculating molecular weights from sedimentation equilibrium experiments performed in 6 M Gu·HCl. (1) Preferential guanidination is minimal, and ϕ' is identical with $\bar{v}$, or 0.725 ml/g. (2) The binding of Gu·HCl to the protein results in a decrease of 0.01 ml/g in ϕ' or, ϕ' is equal to 0.715 ml/g.

Sedimentation velocity experiments were performed using schlieren optics in Kel F single-sector centerpieces. All sedimentation velocity studies were conducted at a temperature of 25°, and a rotor speed of 59,780 rpm.

Intrinsic Viscosity. Viscosity measurements were carried out according to the procedure previously described (Tanford *et al.*, 1967). Solutions for viscosity measurements were prepared from stock solutions of transferrin, the concentrations of which were determined by dry weight.

The intrinsic viscosity of reduced transferrin in 6 M Gu·HCl was treated in terms of the equation of Tanford *et al.* (1967) who have applied the general equation for the viscosity of linear random coils to reduced protein polypeptide chains in 6 M Gu·HCl. In this equation

$$[\eta]M_0 = 77n^{0.666}$$

$[\eta]$ is the observed intrinsic viscosity of the linear randomly coiled polypeptide chain, M_0 is the mean residue molecular weight of the protein which can be calculated from its chemical composition, and n is the number of amino acid residues in the polypeptide chain. A mean residue weight of 116 was calculated for transferrin by division of the native molecular weight (including carbohydrate content) by the number of amino acid residues per molecule.

The molecular weight of the native protein was calculated from its respective sedimentation velocity and intrinsic viscosity using the equation of Scheraga and Mandelkern (1953).

$$M^{2/3} = \frac{Ns[\eta]^{1/3}\eta_0}{\beta(1 - \bar{v}\rho)}$$

The value chosen for β was 2.12×10^6. This value is most appropriate for compact globular proteins.

Gel Filtration. Gel filtration studies were conducted according to the method of Fish *et al.* (1969). The distribution coefficients, K_d, of the transferrin polypeptide chain were obtained from the elution positions from calibrated 6 and 4% agarose columns, prepared, and operated in 6 M Gu·HCl. Elution positions were monitored by absorbance at 280 mμ and by turbidimetric analysis. The apparent molecular weights were determined by comparison of the observed K_d values with a calibration curve of log molecular weight *vs.* K_d prepared with protein polypeptide chains of known molecular weight.

Chemical Studies. Transferrin samples were hydrolyzed *in vacuo* in 6 N HCl at 106° from 24 to 72 hr. Amino acid analyses were performed on a Beckman Spinco amino acid analyzer. Tryptophan was estimated by the method of Edelhoch (1967). Cysteine was determined as cysteic acid using the method of Hirs (1967a), and as carboxymethylcysteine, using the method of Hirs (1967b).

Fingerprint studies were carried out by the method of Stears *et al.* (1965). Transferrin samples were reduced, or reduced and carboxymethylated in 6 M Gu·HCl, dialyzed exhaustively *vs.* 10^{-3} M HCl, and heat denatured. Hydrolysis by TPCK-trypsin was carried out from 5 to 24 hr at 37° in 0.2 M NH_4HCO_3.[3] Mapping was accomplished by descending chromatography in butanol–acetic acid–water (4:1:5) or pyridine–butanol–acetic acid–water (60:90:18:72), and high-voltage electrophoresis in pyridine–acetic acid–water (1:10:289), pH 3.6. Peptides were located by a ninhydrin spray. Specific staining for tyrosine, tryptophan, and histidine was accomplished using the procedures described by Smith (1958).

Qualitative amino-terminal studies were performed using the dansyl method (Woods and Wang, 1967). The cyanate method of Stark and Smyth (1963) was used for the quantitative amino-terminal analyses.

Results

Sedimentation Equilibrium. The meniscus depletion method was applied to a 0.01% solution of transferrin in 0.1 M NaCl, pH 6.5. The rotor speed in this experiment was 21,740 rpm, and the final photograph was taken 22 hr after reaching speed. The graphical interpretation of the data in terms of a plot of the log of the Rayleigh fringe displacement, Y, *vs.* the square of the distance from the center of rotation, r^2, is shown in

[3] Samples which were simply reduced were hydrolyzed in 0.1 M $(NH_4)HCO_3$–0.01 M 2-mercaptoethanol to prevent the possibility of reoxidation of cysteine.

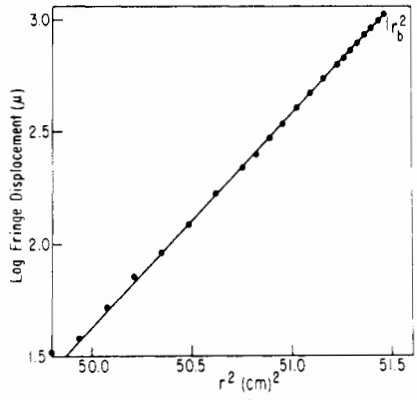

FIGURE 1: Plot of log fringe displacement, Y, *vs.* square of distance from center of rotation, r^2. Sedimentation equilibrium data for a solution of native transferrin (0.01%) in 0.1 M NaCl, pH 6.5. The experiment was carried out for 22 hr at 21,740 rpm. The line is drawn from a least-squares analysis which includes all fringe displacements greater than 100 μ.

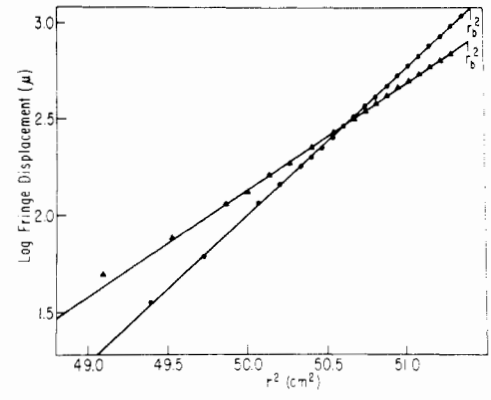

FIGURE 2: Plot of log fringe displacement, Y, *vs.* square from center of rotation, r^2. Sedimentation equilibrium data for a solution of reduced transferrin (0.02%) in 6.2 M of distance Gu·HCl–0.1 M 2-mercaptoethanol, pH 6.0. The data are for experiments performed at 25,980 rpm (●), and 21,740 rpm (▲). The lines are drawn from least-squares analysis which include all fringe displacements greater than 100 μ.

Figure 1. Under these experimental conditions, the material is apparently homogeneous, and the molecular weight is 76,600. This value is in good agreement with the molecular weight of 73,000–76,000 based on sedimentation equilibrium and osmotic pressure measurements, reported by Roberts *et al.* (1966), and the sedimentation equilibrium value of 77,000 reported by Greene and Feeney (1968). The apparent discrepancy between these data and the value of 93,000 reported by Bezkorovainy and Grolich (1967) from sedimentation and diffusion data, can be partly explained by their use of an apparent effective ϕ' of 0.758 ml/g. With the value of ϕ' used in this paper, their molecular weight would become 82,000.

We have taken our value of 76,600 to be the molecular weight of native transferrin, and this value will be used throughout the text.[4]

To determine if the molecular weight of transferrin would decrease under reducing, denaturing conditions, sedimentation equilibrium studies were performed on reduced, and reduced carboxymethylated transferrin in 6 M Gu·HCl at pH 6.0.

Figure 2 presents the graphical data for two sedimentation equilibrium experiments performed on a 0.02% solution of reduced transferrin in 6.2 M Gu·HCl–0.2 M 2-mercaptoethanol at 21,740 rpm and 25,980 rpm. Both plots are linear to the bottom of the cell and, assuming that ϕ' equals $\bar{v}$, yield molecular weights of 77,900 and 74,700, respectively. A ϕ' value of 0.715 ml/g (a decrease of 0.01 in $\bar{v}$) would lead to molecular weight values of 73,100 and 70,100, respectively. Table I gives the values of the molecular weights obtained in five separate sedimentation equilibrium experiments performed in 6 M Gu·HCl.

Sedimentation Velocity. The sedimentation velocities of apotransferrin were measured using solutions of apotransferrin in 0.1 M NaCl, pH 6.0, at 25°. The values corrected to standard conditions are plotted in Figure 3. The $s^0_{20,w}$ obtained from the intercept was 5.25×10^{-13} cm/sec, and the concentration dependence k was 5.25×10^{-12} cm^4/g sec, calculated according to

$$s_{20,w} = s^0_{20,w} - kc$$

where c is the protein concentration in g/cm^3.

Bezkorovainy (1966a) has reported that under almost the same conditions used for our measurement of the s values of apotransferrin, transferrin sediments with an asymmetric boundary due to aggregation. No asymmetry nor any other evidence of heterogeneity was detected in our sedimentation patterns for apotransferrin. Furthermore, the concentration dependence in our study was the same as or slightly higher than that found by other workers who reported a similar $s^0_{20,w}$; this is contrary to what would be expected if aggregation were taking place.

Although the reported $s^0_{20,w}$ values for transferrin vary from 4.9 to 6.1 S, the values higher than 5.4 S can probably be disregarded because of the aggregation. Charlwood (1963) first used this explanation for the difference between his values of 5.1 and 5.2 S for monkey, rat, and human transferrin as well as for the higher values of other workers (5.5 S or greater) which he listed. Bezkorovainy (1966a) reported several conditions that led to aggregation of transferrin. Under conditions where no aggregation occurred, he gave $s^0_{20,w}$ values of 5.3–5.4 S for transferrin and apotransferrin (Bezkorovainy, 1966b). Roberts *et al.* (1966) have reported the lowest value, 4.92 ± 0.12 S. A reasonable range for $s^0_{20,w}$ is 5.2 ± 0.2 S.

Intrinsic Viscosity. The intrinsic viscosity of both native ferritransferrin and native apotransferrin in 0.1 N NaCl pH 6.0, was 4.0 ml/g (Figure 4A). This value is typical of native globular proteins. Combination of this value for $[\eta]$ with our value for $s^0_{20,w}$ in the Scheraga–Mandelkern equation results in a calculated value for the molecular weight of native human transferrin of 79,300.

Figure 4B presents the intrinsic viscosity data for transferrin in 6 M Gu·HCl with disulfide bonds intact (17 ml/g),

[4] Preliminary data from this laboratory, reported in a footnote by Leibman and Aisen (1967), gave a value of 82,000 for the native molecular weight of transferrin. Careful reexamination of the plates on which these results were based indicated the presence of higher molecular weight aggregates.

TABLE I: Molecular Weights of Human Serum Transferrin in 6 M Gu·HCl.

Protein Concn (%)	Rotor Speed (rpm)	Molecular Weight ϕ_1' (0.725 ml/g)	Molecular Weight ϕ_2' (0.715 ml/g)
0.01	25,980	79,000	74,200
0.02	25,980	74,700	70,100
0.02	21,740	77,900	73,100
0.01	29,500	74,400	69,900
0.02[a]	25,980	75,500	70,700
	Average	76,300	71,600

[a] Reduced, carboxymethylated transferrin.

and reduced transferrin in 6 M Gu·HCl–0.1 M 2-mercaptoethanol (50.8 ml/g). The dramatic increase in intrinsic viscosity upon reduction of transferrin indicates the molecule is highly cross-linked by disulfide bridges.

When the value of the intrinsic viscosity and mean residue weight of reduced transferrin in 6 M Gu·HCl are substituted into the equation of Tanford *et al.* (1967), a value for n is obtained which is commensurate with a linear randomly coiled polypeptide chain of 674 amino acid residues, and a molecular weight of 78,300, This molecular weight is consistent with the results obtained from the analytical ultracentrifuge, and virtually precludes the possibility of two chains connected by a cross-link which is resistant to cleavage under the experimental conditions employed. Unless such a crosslink were very close to the terminii of the chains, the viscosity would be much lower than that of a continuous linear chain.

Gel Filtration in 6 M Gu·HCl. The molecular weights of the components present in reduced and fully carboxymethylated transferrin samples were examined by gel filtration in 6 M Gu·HCl using the method of Fish *et al.* (1969).

When reduced, carboxymethylated transferrin was subjected to gel filtration in 4 or 6% agarose in the presence of 6 M Gu·HCl, it eluted as a single component at a position ahead of bovine serum albumin (mol wt 69,000). In no case were any smaller components detected.

The accuracy of molecular weight determinations for linear random coils of molecular weight greater than 70,000 is not optimal on 6% agarose because of the proximity of elution of such materials to the column void volume. For polypeptide chains in the molecular weight range of transferrin, molecular weight measurements on 4% agarose are somewhat imprecise because of the paucity of calibrating standards of molecular weight greater than 80,000. However, in every case, reduced, carboxymethylated transferrin eluted at a position consistent with a molecular weight for a linear random coil of 70,000 to 80,000.

Normally, reduction of protein disulfide bonds in 6 M Gu·HCl–0.1 M 2-mercaptoethanol, pH 8.6, is complete in 4 hr. In order to be certain that all disulfide bonds were reduced, more drastic reduction conditions were employed. Transferrin in 6 M Gu·HCl was subjected to 0.7 M 2-mercaptoethanol at pH 10.5 for 48 hr. Even under these conditions, the protein

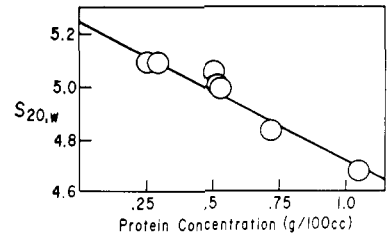

FIGURE 3: Sedimentation velocity of apotransferrin in 0.1 M NaCl, pH 6.0, at 25°.

eluted as a single peak with an elution position corresponding to a molecular weight of 70,000–80,000. No trace of any component with lower molecular weight was observed. A single gel filtration experiment was also performed on a sample of transferrin which had been oxidized with performic acid. Oxidation of transferrin led to no change in the observed distribution coefficient of the protein. The fact that both oxidation and reduction of the protein resulted in the same gel filtration behavior indicates that both methods cleaved the same interchain bonds.

Table II presents a summary of the molecular weight data obtained for transferrin by the methods described in this section.

Chemical Studies

Amino Acid Composition. Transferrin contains about 6.2% carbohydrate (Jamieson, 1965). Therefore, we have calculated our amino acid compositions based on an amino acid content weight of 71,850. The mean values obtained from amino acid analysis of 24-, 48-, and 72-hr hydrolysates of transferrin are presented in Table III. For comparison purposes, the amino acid composition data of Bezkorovainy *et al.* (1968)

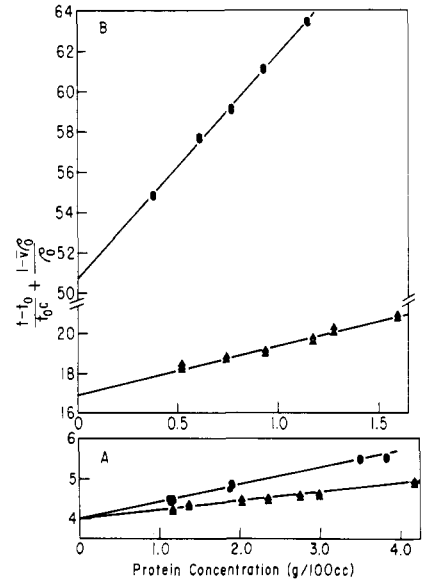

FIGURE 4: Viscosity measurements at 25°. (A) Native transferrin in 0.1 M NaCl, pH 6.0 (▲) transferrin; (●) apotransferrin. (B) (▲) transferrin with disulfide bonds intact in 6 M Gu·HCl; (●) reduced transferrin in 6 M Gu·HCl–0.1 M 2-mercaptoethanol.

TABLE II: Summary of Molecular Weight Measurements on Human Serum Transferrin.

Method	Mol Wt
Sed. equil in 0.1 M NaCl, pH 7.0	76,600
Sed. vel and [η] in 0.1 M NaCl, pH 6.0	79,300
Sed. equil in 6 M Gu·HCl–0·1 M RSH	76,800 ± 3,000[a]
Sed. equil of reduced and carboxymethylated-protein in 6 M Gu·HCl	75,500
Intrinsic viscosity in 6 M Gu·HCl–0.1 M RSH	78,300
Gel filtration of reduced and carboxymethylated-protein in 6 M Gu·HCl	70,000–80,000 with *no* smaller components

[a] 71,600 assuming ϕ' is decreased by 0.01 ml/g in this solution.

TABLE III: Amino Acid Composition of Human Serum Transferrin.

Amino Acid	This Study, Residues/ 76,600 g[a]	Bezkorovainy *et al.* (1968), Residues/ 76,600 g
Lys	51	58
His	19	16
Arg	24	24
Asp	81	74
Thr[b]	30	26
Ser[b]	40	35
Glu	58	55
Pro	30	28
Gly	54	46
Ala	59	53
Cys[c]	35 (32[d])	32
Val	43	34
Met	4	8
Ile	14	14
Leu	57	56
Tyr	24	23
Phe	28	26
Trp[e]	9	7

[a] Residues based on molecular weight less 6% carbohydrate. [b] By extrapolation to zero time of hydrolysis. [c] As cysteic acid. [d] As carboxymethyl-Cys. [e] By spectrophotometric method of Edelhoch (1967).

for transferrin are also presented, normalized to an amino acid composition weight of 71,850.

It should be noted that a slight discrepancy exists between our carboxymethylcysteine and cysteic acid values. Performic acid oxidation of transferrin gave a value of 35 cysteines/molecule while carboxymethylation data gave 32 cysteines/molecule. We do not feel that this difference is significant in terms of the relative degree of disulfide-bond cleavage by the oxidation or reduction procedures, but rather a reflection of the precision of the methods of analysis.

A basic area of disagreement between our data and that of Bezkorovainy *et al.* (1968) is in the relative amount of methionine. At present we have no explanation for this discrepancy.

Fingerprint Studies. Transferrin contains a total of 75 lysine and arginine residues per molecule. If the structure is a single polypeptide chain with no repeating sequences, the molecule should be cleaved at 75 sites with the production of 76 peptides.

Fingerprint studies were performed in a variety of solvents and under varying conditions of hydrolysis to preclude the possibility of artifacts. Even TPCK-blocked trypsin may possess some residual chymotryptic activity (Kostka and Carpenter, 1964). To prevent the possibility of the formation of anomalous chymotryptic peptides, hydrolyses were carried out for various lengths of time. Hydrolyses were performed on both reduced and reduced carboxymethylated transferrin to minimize the possibility of spurious peptides due either to the formation of carboxymethyllysine, -histidine, and -methionine peptides,[5] or to the reformation of disulfide bridges in the noncarboxymethylated samples. Chromatography of the tryptic hydrolysates was carried out in either of two solvent systems to minimize peptide overlap in a given solvent, thus resulting in a smaller number of observed peptides.

Figure 5 presents two typical examples of the tryptic peptide maps obtained in this study. The numbers of ninhydrin-positive peptides detected in these experiments were as follows: 60–70 peptides for the 24-hr tryptic hydrolysis of reduced carboxymethylated transferrin, 58–70 peptides for the 24-hr tryptic hydrolysis of reduced transferrin, and 54–60 peptides for the 5-hr tryptic hydrolyses of reduced transferrin.

Specific sprays for tryptophan (Ehrlich), tyrosine (Pauley, α-nitrosonaphthol), and histidine (Pauley) were employed to detect the number of peptides containing these residues. Table IV shows the results of these experiments. The number of peptides expected if transferrin were a single, nonrepeating chain is also shown.

Preliminary fingerprinting studies were conducted on the products obtained by cyanogen bromide cleavage of reduced carboxymethylated transferrin. The poor solubility of the cyanogen bromide cleavage products hampered the mapping operation, but five ninhydrin-positive peptides were observed.

Amino-Terminal Studies. Qualitative amino-terminal amino acid determinations by the dansyl chloride method showed valine to be the only amino-terminal amino acid. The method of Stark and Smyth (1963) was then employed for quantitative amino-terminal studies.

Four independent quantitative determinations gave an average of 0.5 ± 0.02 mole of valine/mole of transferrin. One-half mole of end group is of course impossible but hemoglobin, which was used as a control, also characteristically gives low values for amino-terminal valine because of a Val–

[5] None of these modified residues were observed, however, when samples of the trypsin-hydrolyzed reduced carboxymethylated protein were subjected to amino acid analysis.

TABLE IV: Number of Peptides Observed on Tryptic Peptide Maps of Human Serum Transferrin.

Stain	Observed	Expected
Ninhydrin	55–70	76
Pauly (His)	12–15	19
Pauly or nitroso-naphthol (Tyr)	13–14	24
Ehrlich (Trp)	6	9

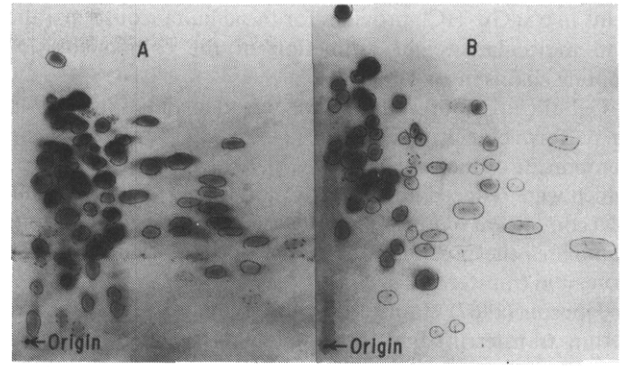

FIGURE 5: TPCK-trypsin fingerprints. (A) Reduced carboxymethylated transferrin: hydrolysis was carried out for 24 hr at 37° in 0.2 M NH_4CO_3. Mapping was accomplished by descending paper chromatography in the horizontal direction in pyridine–butanol–acetic acid–H_2O (60:90:18:72), and high-voltage electrophoresis in pyridine–acetic acid–H_2O (1:10:289) in the vertical direction. (B) Reduced transferrin: hydrolysis was carried out for 5 hr at 37° in 0.2 M NH_4HCO_3. Mapping was accomplished by descending paper chromatography in butanol–acetic acid–H_2O (4:1:5) in the horizontal direction and high-voltage electrophoresis in pyridine–acetic acid–H_2O (1:10:289) in the vertical direction.

Leu bond at the α-chain terminus which is particularly difficult to hydrolyze (Bradshaw *et al.*, 1965).

Although our quantitative amino-terminal results offer no conclusive evidence for the number of polypeptide chains in transferrin, they certainly do not suggest the existence of two chains in the molecule.

Discussion

The sedimentation equilibrium data reported in this paper indicate that the molecular weight of native transferrin is unchanged upon solvation of the reduced protein in 6 M Gu·HCl–0.1 M 2-mercaptoethanol, or the reduced carboxymethylated protein in 6 M Gu·HCl. This result is contrary to the report of Jeppson (1967) (approach to equilibrium) that reduced, carboxymethylated, or performic acid oxidized transferrin is dissociated in 8 M urea into two subunits of similar molecular weight. Our sedimentation equilibrium data are consistent with the reports of Bezkorovainy and Grohlich (1967) (sedimentation diffusion), and Greene and Feeney (1968) (sedimentation velocity) that transferrin is composed of a single polypeptide chain.

One cannot discount the discrepancy between our data and that of Jeppson on the basis of different solvents (6 M Gu·HCl *vs.* 8 M urea), since it has been well established that 6 M Gu·HCl is a more effective dissociating agent than 8 M urea (Tanford, 1968). The observed intrinsic viscosity for reduced transferrin in 6 M Gu·HCl is consistent with complete disruption of all noncovalent bonds and preliminary optical rotatory dispersion measurements performed in 6 M Gu·HCl also indicate that the protein secondary and tertiary structure is entirely disrupted in this solvent.

While the sedimentation equilibrium data presented here suggest that transferrin consists of only a single chain, they do not by themselves preclude two alternate possibilities to a single polypeptide chain structure. First, that transferrin is composed of multiple polypeptide chains held together by cross-linkages which are impervious to the solvent system. That is, transferrin might possess a cross-link of unknown nature which remains intact after reduction and carboxymethylation of the protein. Second, that transferrin is composed of two polypeptide chains, one of which is a relatively small peptide. These two possibilities were evaluated by viscosity measurements and gel filtration of the reduced protein in 6 M Gu·HCl.

Since the sedimentation equilibrium technique measures mass directly, it cannot distinguish between a single polypeptide chain of given mass, and a cross-linked set of polypeptide chains of the same cumulative mass. The calculation of a polypeptide chain's molecular weight from intrinsic viscosity measurements depends upon the molecule assuming linear random coil dimensions in a denaturing solvent. The presence of inter- or intrachain cross-links in a fully denatured polypeptide chain(s) would prevent linear random coil behavior, and lead to a low value for the intrinsic viscosity thus giving an erroneous value for the molecular weight.

An intrinsic viscosity of 50.8 ml/g was observed for reduced transferrin in 6 M Gu·HCl–0.1 M 2-mercaptoethanol. This value is somewhat lower than the value of 59.8 ml/g determined by Bezkorovainy and Grohlich (1967) in the same solvent. Substitution of our intrinsic viscosity value together with the mean residue weight of transferrin into the viscosity equation of Tanford *et al.* (1967) gives a value of 78,300 for the mol wt. This value is in good agreement with the true mass value of 76,600 determined in the analytical ultracentrifuge and almost precludes the possibility of a series of crosslinked polypeptide chains. It should be noted, however, that a similar intrinsic viscosity would be observed if transferrin were composed of two chains cross-linked at or near their terminii.

The presence of a small molecular weight (5,000–10,000) polypeptide chain in transferrin could possibly escape detection in sedimentation equilibrium studies. The uncertainty in the assignment of ϕ' in denaturing solvents and the inherent error in sedimentation equilibrium experiments ($\simeq \pm 5\%$) could combine to exclude detection of such a component.

Gel filtration of reduced protein polypeptide chains in 6 M Gu·HCl provides an unambiguous technique for the evaluation of the molecular weight of the component polypeptide chains in a protein (Fish *et al.*, 1969). For reduced proteins in 6 M Gu·HCl, a protein's native shape is not a factor and the relative elution position is a function of chain length. Thus, a calibrated gel filtration column, operated with reduced pro-

teins in 6 M Gu·HCl, provides for the simultaneous separation and molecular weight estimation of the component polypeptide chains in a protein.[6]

Gel filtration of reduced carboxymethylated transferrin, or performic acid oxidized in 6 M Gu·HCl revealed a single component of molecular weight 70,000–80,000. Even samples which were reduced under drastic conditions gave no evidence of a component of lower molecular weight. This result virtually eliminates the possibility of a small polypeptide chain component in transferrin.

Jeppson (1967) (transferrin) and Williams (1962) (chicken serum transferrin) have reported that tryptic hydrolysis of the protein results in far fewer than the expected number of peptides one would expect based on the Lys-Arg content of these proteins.

We have prepared tryptic peptide maps for both reduced, and reduced carboxymethylated transferrin. Hydrolysis times and mapping solvent conditions were varied in order to reduce the possibility of artifacts. However, in no case were we able to detect fewer than 54 ninhydrin-positive peptides. Amino acid composition studies indicate that tryptic cleavage of transferrin should result in 76 peptides if the structure is a single polypeptide chain of nonrepeating sequence. The detection of 70% of the expected tryptic peptides is consistent with similar observations for tyrosine- (54%), tryptophan- (67%), and histidine- (63%) containing peptides. Our peptide mapping results are similar to those reported by Baker *et al.* (1968) for tryptic peptide maps of rabbit serum transferrin.

The observation of fewer than the expected number of ninhydrin-positive and specific amino acid containing peptides can be explained in either of two fashions. The first of these is that due to the difficulties in resolving a large number of peptides low yields of ninhydrin-positive peptides are possible, and low yields of specific amino acid containing peptides may occur owing to the repetitive occurrence of the same residue in a given peptide. The second possibility is that some portions of the sequence of the protein occur in duplicate. This hypothesis is reinforced by the occurrence of duplicate iron binding sites, and carbohydrate-linked peptides in the molecule. The possibility then exists that the structural gene for transferrin underwent duplication, and subsequent fusion in its evolutionary history.

[6] The actual parameter measured in gel filtration studies of linear random coils is entirely analogous to that measured in intrinsic viscosity studies. However, while the latter measures an average property for all components, gel filtration provides a specific value for the molecular weight of each separable component.

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