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# Physical Evidence for Transferrins as Single Polypeptide Chains*

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ABSTRACT: Physical methods were used to evaluate the possibility that the two iron binding sites of transferrins are located in two subunits of the proteins. The disulfide bonds in chicken ovotransferrin, human serum transferrin, and rabbit serum transferrin were reduced, and the resulting sulfhydryls were carboxymethylated. These carboxymethylated transferrins were compared ultracentrifugally with similarly produced carboxymethylated derivatives of bovine serum albumin and porcine pepsin.

The $s_{20}$ values of these derivatives observed in 8 M urea and in 6 M guanidine hydrochloride were proportional to the molecular weights of the native proteins as determined by sedimentation equilibrium. These results indicated that the transferrins had not been dissociated by reduction. The modified proteins in 8 M urea were analyzed using the relation $s/(1 - \bar{v}\rho) \propto M^{1/2}$ as predicted from theory for random coils. The minimum weight of the polypeptide chain of ovotransferrin as estimated from this relation is equivalent to its molecular weight. All three transferrins, therefore, appear to be monomers.

The transferrins are a group of homologous, iron binding glycoproteins present in vertebrate blood, mammalian milk, and avian egg whites (Feeney and Komatsu, 1966). The serum transferrins have been implicated in the transfer of iron from storage areas to immature red blood cells (Jandl *et al.*, 1959). Transferrins from several different sources have similar molecular sizes (mol wt 70,000–90,000), similar iron binding properties ($K_{diss} \simeq 10^{-29}$), and similar amino acid composition (Feeney and Komatsu, 1966; Osuga and Feeney, 1968). The two iron binding sites are apparently equivalent and noninteracting (Aisen *et al.*, 1966). The iron binding ligands of the proteins are contributed by amino acid side chains (Fraenkel-Conrat and Feeney, 1950; Windle *et al.*, 1963), and the characteristic color is dependent on bicarbonate binding (Aisen *et al.*, 1967).

Tryptic peptide maps of chicken ovotransferrin (OT)[1] (Williams, 1962) and human serum transferrin (HST) (Jeppsson, 1967) show much smaller numbers of spots than would be predicted from their lysine and arginine content. HST apparently contains two similar oligosaccharides (Jamieson, 1965). These data,

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[1] Abbreviations used that are not listed in *Biochemistry 5*, 1445 (1966) are: OT, chicken ovotransferrin; HST, human serum transferrin; RST, rabbit serum transferrin; RCM, reduced carboxymethylated; BSA, bovine serum albumin.

the low number of peptides, the presence of two iron binding sites, and the observed molecular weights would, *a priori*, implicate transferrins as proteins consisting of two similar or identical subunits. In fact, two subunits have been reported for HST on the basis of sedimentation velocity studies on the RCM protein (Jeppsson and Sjöquist, 1964). However, only one N-terminal amino acid per mole has been reported for HST and OT and none for human lactotransferrin (Feeney and Komatsu, 1966). In the present investigation, we have attempted to determine the presence or absence of subunit structure in transferrins by physical methods.

The sedimentation behavior of reduced-carboxymethylated preparations of OT, HST, and rabbit serum transferrin (RST) has been compared with those of bovine serum albumin (BSA) and those of pepsin in 8 M urea or 6 M guanidine hydrochloride. The RCM proteins are expected to assume random coil form in these denaturing solvents (Urnes and Doty, 1961; Tanford *et al.*, 1967a), and their sedimentation behavior should therefore be a predictable function of their molecular weight in solution. The results are consistent with a model of a single-chain polypeptide for all the transferrins.[2]

## Materials

Chicken OT prepared according to Warner (1953) was further purified by DEAE-cellulose chromatography (Clark *et al.*, 1963). HST was prepared from Cohn fraction IV by chromatography on DEAE-cellulose first with a gradient of ionic strength (Gordon and Louis, 1963) and then with a pH gradient (Komatsu and Feeney, 1967).

RST was a gift from Dr. Evan H. Morgan. BSA (two-times crystallized), guanidine hydrochloride, and 2-mercaptoethanol were obtained from Sigma Co. Pepsin (three-times crystalline) and iodoacetic acid were obtained from Nutritional Biochemical Co. Tris-HCl was obtained from Eastman Kodak Co. Urea (Baker analyzed reagent) and guanidine hydrochloride were recrystallized from water before use.

## Experimental Section

Molecular weights of OT and RST were determined at 0.2% protein concentration in 0.05 M ($\Gamma = 0.09$; $\rho = 1.004$) (pH 6.5) sodium phosphate buffer by short-column sedimentation equilibrium at 20° and 8766 rpm. Molecular weight values were calculated from the slope of plots of ln $j(r)$ *vs.* $r^2$ (where $j$, the fringe number, is proportional to $c$, the concentration) according to the equation $\mathrm{d} \ln c/\mathrm{d}r = w^2M(1 - \bar{v}\rho)r/2RT$ (Svedberg and Pedersen, 1940). The absolute fringe number at the meniscus was determined from an equation equivalent to

$$C_m = C_0 - \frac{x_b^2(C_b - C_m) - \int_{x_m}^{x_b} x^2 \mathrm{d}c}{x_b^2 - x_m^2}$$

(where $c \propto j$) by a numerical integration method (Van Holde, 1967). Values used for $\bar{v}$ for the native proteins were: OT, 0.732 (Fuller and Briggs, 1956); HST, 0.725 (Oncley *et al.*, 1947); RST, 0.725 by similarity to HST; and pepsin, 0.726 (calculated from amino acid composition by Williams and Rajagopalan, 1966). Values for the RCM proteins were calculated from amino acid composition according to Cohn and Edsall (1943) assigning a value of 0.60 as the $\bar{v}$ for the *S*-carboxymethylcysteinyl residue.[3] These values are: RCM-OT, 0.729; RCM-BSA, 0.729; and RCM-pepsin, 0.725, which are decreases of 0.003, 0.005, and 0.001 cc/g from the native proteins. The RST appeared homogenous from the linearity of its plot, but a small amount of a heavier component in the OT was indicated by a slight upward curvature of its plot toward the cell bottom. The molecular weights from duplicate determinations were: OT, 75,190 and 75,900 (rounded to 76,000); RST, 77,150 and 76,330 (rounded to 77,000).

The procedure for reduction and carboxymethylation was modified from that of Crestfield *et al.* (1963). A typical experiment was as follows. Transferrin (10 mg) was dissolved in 2 ml of 8 M urea (or 6 M guanidine hydrochloride)–0.3 M $O_2$-free Tris-HCl buffer at pH 8.6. The resulting protein concentration was $\sim 6.5 \times 10^{-5}$ M. 2-Mercaptoethanol (0.068 ml) was added to a concentration of 0.5 M so that its excess over potential SH groups in the proteins was approximately 1000-fold. The reduction was allowed to proceed in an $N_2$ atmosphere in sealed, plastic-capped vials for 12 hr at 45° with magnetic stirring. At the end of this period the vials were cooled to room temperature, and 227 mg of iodoacetate (a 10% excess based on 2-mercaptoethanol) in 0.6 ml of 1.0 M NaOH was added dropwise with a hypodermic syringe through the cap of the vials. After 30 min the reaction mixture (about pH 8.5) was dialyzed against deionized water, and the resulting suspension was lyophilized. The extent of modification was determined by amino acid analysis of 24-hr 6 M HCl hydrolysates with a Technicon amino acid analyzer (Table I). With the exception of BSA, the residual half-cystine content was reduced to less than one per mole, eliminating the possibility of peptide-linking disulfide bridges.

Sedimentation coefficients ($s_{20,obsd}$) were determined from the slopes of plots of log $x$ *vs.* $t$ (Schachman, 1959) at speeds of 56,100 or 59,780 rpm at 20° in 0.1 M

[2] During the terminal phases of this work we found a reference to unpublished work of Parker *et al.* (Bearn and Parker, 1966) concerning sedimentation equilibrium and gel filtration studies on HST, also concluding that no subunit structure was present. In addition, reference has been made in *Arch. Biochem. Biophys.* (1967) by Leibman and Aisen, to studies in progress by A. C. Cox and C. Tanford who reached the same conclusion.

[3] Calculated by addition of the atomic weight and molal volume of sulfur to that of the glutamyl residue. The molal volume contribution of sulfur was taken as the difference between the molal volumes of methionyl- and α-aminovaleryl residues.

TABLE I: CM-Cysteine Recovery and Residual Half-Cysteine in RCM Proteins.[a]

| Protein | Mol Wt | Native Cys(1/2)/ Mole | RCM Protein[b] CM-Cys[l]/ Mole | RCM Protein[b] Cys(1/2)/ Mole | Possible S–S Bonds Based on CM-Cys[l]/ Mole | Possible S–S Bonds Based on Cys(1/2)/ Mole | Reduction Solvent |
|---|---|---|---|---|---|---|---|
| BSA | 66,000[c] | 36[d] | 32.7 | 1.7 | 1–2 | 1 | Urea |
| | | | 32.0 | 2.7 | 2 | 1–2 | Gu·HCl[i] |
| OT | 76,000[e] | 25[f] | 23.7 | 0.5 | 0 | 0 | Urea |
| | | | 22.9 | 0.4 | 1 | 0 | Gu·HCl |
| RST | 77,000[e] | 30[e] | 29.2 | 0.2 | 0 | 0 | Gu·HCl |
| HST | 76,000[g] | 33[h] | 33.3 | 0.5 | 0 | 0 | Gu·HCl |
| PEP | 32,700[j] | 6[k] | 5.0 | 0.6 | 0 | 0 | Urea |

[a] See text for procedure. [b] The results by CM-Cys per mole and Cys(1/2) per mole are not strictly additive. [c] Shulman (1953). [d] Spahr and Edsall (1964). [e] Values from this work. [f] Osuga and Feeney (1968). [g] Roberts *et al.* (1966). [h] Based on Parker and Bearn (1962). [i] Guanidine hydrochloride. [j] Williams and Rajagopalan (1966). [k] Rajagopalan *et al.* (1966). [l] Carboxymethylcysteine.

(pH 8.6) Tris-HCl buffer containing 8 M urea (Table II) or 6 M guanidine hydrochloride (Table III). Solvent density values were estimated from data of Kawahara and Tanford (1966). They are: 1.116 g/cc for the 8 M urea, 0.1 M (pH 8.6) Tris system, and 1.148 g/cc for the 6 M guanidine hydrochloride–0.1 M (pH 8.6) Tris system. Protein concentrations were 0.5%.

To eliminate the possibility that OT consisted of two peptide chains which could associate noncovalently, sedimentation was studied in both 8 M urea and 6 M guanidine hydrochloride. These denaturing solvents generally minimize all noncovalent interaction in proteins and allow them to assume random coil form (Urnes and Doty, 1961; Tanford *et al.*, 1967a). In applying this principle, we assume that reduced-alkylated protein chains in random coil form will exhibit sedimentation behavior which is a function only of their particle weight (chain length). In analyzing OT, we consider two possibilities: (a) a single chain of

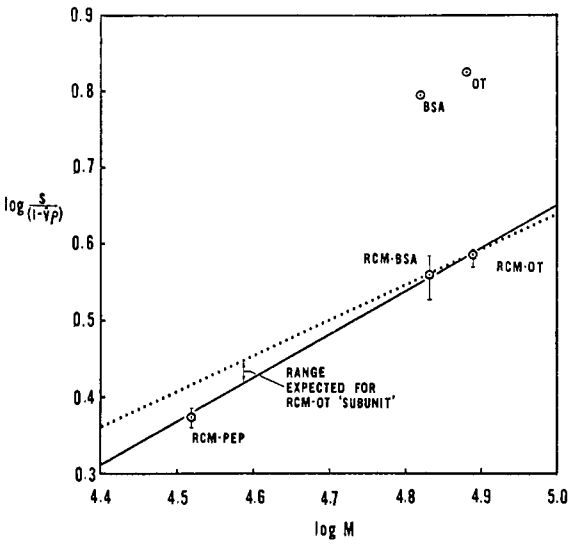


FIGURE 1: Relation of sedimentation coefficients to molecular weights of RCM proteins in 8 M urea, and the behavior of nonreduced BSA and OT for comparison. The vertical bars indicate the range of $s_{20}$ values observed. The dotted line represents the "theoretical" slope with BSA as a reference point and the solid line our experimental slope. The double-headed arrow indicates the range of $s/(1 - \bar{v}\rho)$ values expected for a half-molecule of OT.

TABLE II: Sedimentation in 8 M Urea.

| Protein | Particle Wt | $s_{20,obsd}$ | $\bar{v}$ Assumed |
|---|---|---|---|
| RCM-OT | 77,500 | 0.727 | 0.729[b] |
| | 38,750[a] | 0.725 | |
| | | 0.698 | |
| | | 0.730 | |
| | | Av 0.72 | |
| RCM-BSA | 67,800[a] | 0.720 | |
| | | 0.683 | 0.729[b] |
| | | 0.679 | |
| | | 0.630 | |
| | | Av 0.68 | |
| RCM-PEP | 33,000[a] | 0.436 | |
| | | 0.463 | 0.725[b] |
| | | Av 0.45 | |
| OT | 76,000 | 1.23 | 0.732 |
| BSA | 66,000 | 1.14 | 0.734 |

[a] Complete carboxymethylation would increase molecular weights to these values. [b] Values for the RCM proteins were calculated from the amino acid compositions, assigning a value of 0.60 as the $\bar{v}$ contribution of the *S*-carboxymethylcysteinyl residues (see Experimental Section).

~76,000 mol wt, and (b) two associated chains of ~38,000 mol wt each. We have compared the sedimentation of RCM OT in 8 M urea to that of RCM BSA (mol wt ~67,800) and RCM pepsin (mol wt ~33,000). BSA and pepsin are single-chain polypeptides. A single-chain RCM OT of ~77,500 mol wt should have an $s$ value quite close to that of RCM BSA. A "subunit" OT would yield two RCM chains of ~38,750 mol wt. These RCM chains should separate in 8 M urea, and each chain should have an $s$ value quite close to that of RCM pepsin. Results are shown in Table II. RCM OT has a higher $s_{20,obsd}$ than either of the known single-chain proteins, so its "single"-chain molecular weight must be larger than either of the two RCM reference proteins. The value of 77,500 g/chain must be correct and shows that the molecule is not split into two halves after cleavage of all the disulfide bonds.

The size of RCM OT in 8 M urea may also be evaluated from the following considerations. The frictional coefficients of randomly coiled molecules should be approximately proportional to the square roots of their molecular weights ($f \propto M^{1/2}$).[4] If this proportionality is incorporated into the fundamental equation $s = M(1 - \bar{v}\rho)/Nf$ (see Tanford, 1961), the result is $s \propto M^{1/2}\cdot(1 - \bar{v}\rho)/N$, and it is seen that $s \propto M^{1/2}$. The derivation presented by Tanford is more exact. A plot of log $s^0$ *vs.* log $M$ should be linear, with a slope of ~0.5. Actual values may be less, depending on the nature of the solvent. Tanford *et al.* (1967b), in considering the relation of $s^0$ to chain length, suggested, however, that a more precise relation for proteins, is log $(s^0/(1 - \bar{v}\rho))\cdot \propto 0.5$ log $M$. Figure 1 is a plot from the data of Table II, of log $s_{0.5\%}/(1 - \bar{v}\rho)$ *vs.* log $M$ for RCM OT, RCM BSA, and RCM pepsin. Included are values for nonreduced OT and BSA, to show the effect of the disulfide bonds in restricting conformation. No correction was made for the effect of urea on $\bar{v}$. However, Kielley and Harrington (1960) found that $\bar{v}$ values measured in 5 M guanidine hydrochloride were lowered by ~0.01 cc/g than values in dilute salt solutions. A correction of this magnitude for the RCM proteins studied here would change the slope of the line by only ~0.01.

For a larger series of proteins in guanidine hydrochloride, Tanford *et al.* (1967b) have reported a log $s^0/(1 - \bar{v}\rho)$ *vs.* log $n$ (chain length) slope of 0.473.

For purposes of argument we assume that Tanford's slope (0.473) approximates the theroetical for proteins. This is the dashed line in Figure 1. The solid line represents our experimental slope, which is 0.57. The apparently high experimental slope may indicate a higher dependence of $s$ on $M$ for RCM proteins in general, due to charge-mediated conformational restrictions making the proteins more rodlike; alternatively it may represent an anomaly in the behavior of RCM pepsin. Additional experiments would be needed to decide. The present data is, however, sufficient to predict that the behavior of an RCM peptide of ~39,000-g particle wt (1/2 OT), and the same assumed $\bar{v}$ as RCM OT, would be included in the range bound by the two lines (experimental and theoretical). Thus the peptide would sediment at ~0.49–0.52 S, instead of the observed 0.72 S.

Therefore, OT does not liberate half-molecules upon reduction and alkylation, and probably consists of one polypeptide chain. The possible one to two disulfide bridges in the RCM BSA are apparently positioned in areas of the molecule where they do not greatly affect its ability to behave as a random coil. A significant restriction of conformation by disulfide bonds would result in an $s$ value intermediate between that expected for random coil and that observed for the more compact nonreduced BSA (see Figure 1). The residual bonds may be due either to incomplete reduction or to some reoxidation.

Two other transferrins (HST and RST) were reduced and alkylated in 6 M guanidine hydrochloride, and their sedimentation properties were compared with those of RCM OT and RCM BSA (Table III). These results indicate that probably all transferrins have a single-chain structure and are not dimers linked noncovalently or covalently by disulfide bridges.

## Discussion

The experimental results demonstrate that the particle size of the transferrins studied, and probably of all transferrins, cannot be lowered by cleavage of their disulfide bonds and solution in 8 M urea or 6 M guanidine hydrochloride.[5] These conditions should eliminate all interactions which might normally serve to associate polypeptide chains in solution. However, the RCM transferrins continue to exhibit sedimentation behavior consistent with a weight of ~78,000 awu/polypeptide chain, as shown by comparison with RCM BSA and RCM pepsin.

The finding of a single-chain structure provides interesting material for speculation, along both genetic and physical lines. On a genetic basis, it appears more

[4] The hydrodynamic behavior of a long-chain random coil molecule is explained by equating it to an "equivalent hydrodynamic sphere" (Flory, 1953). The radius of this "equivalent sphere" is related to the radius of gyration of the random coil in such a way that their frictional coefficients ($f = 6\pi\eta Nr$ from Einstein–Stokes; the radius of the equivalent sphere is $r = \xi_t R_g$, where $R_g$ = radius of gyration, and $\xi_t$ is an empirical constant.) will be equal. The radius of gyration (and thus the frictional coefficient) of the random coil is a function of the square root of the chain length or molecular weight of the molecule.

[5] It is difficult to understand the conclusions of Jeppsson and Sjöquist (1964) reporting two HST subunits. They report $s_{20,w}$ values of 2.5 ("major peak") and 5.0 S ("minor peak") for the products of reduction carboxymethylation of HST; and a single peak of 3.0 S for "a transferrin sample treated only in 8 M urea." These low values are ascribed to subunits. A similar $s_{20,w}$ value of 2.8 S for a solution of 1% ovotransferrin in 8 M urea (pH 10.1) is reported by Glazer and McKenzie (1963). Also, when our $s_{20,obsd}$ values of 1.23 and 1.14 S for OT and BSA, respectively, in 8 M urea, and Tanford *et al.*'s (1967b) $s^0_{25,obsd}$ value of 1.4 S for BSA in 6 M guanidine hydrochloride are converted into 20° and water, the resulting $s_{20,w}$ values are 3.01 S for 0.5% ovotransferrin, 2.8 S for 0.5% BSA, and 3.27 S for BSA at infinite dilution. These low values do not indicate dissociation into subunits. They rather indicate that the molecules are partially unfolded in these solvents, and the resulting increase in frictional resistance causes lowered sedimentation rates.

TABLE III: Sedimentation in 6 M Guanidine Hydrochloride.

| Protein[a] | Particle Wt[b] | $s_{20,obsd}$ |
|---|---|---|
| RCM OT | 77,500 | 0.99 |
| RCM HST | 77,900 | 0.87 |
| RCM RST | 78,700 | 0.94 |
| RCM BSA | 67,800 | 0.75 |

[a] Protein concentrations: 0.4%; buffer, 0.1 M Tris·HCl (pH 8.6) and 6 M guanidine hydrochloride. [b] Calculated to include added carboxymethyl groups.

convenient and more economical in terms of required DNA and mRNA to construct a 76,000 mol wt protein by the joining of two preformed 38,000 mol wt halves than by uninterrupted synthesis of a single chain. Twice as much DNA (and mRNA) would be required for the large single chain. The analyses of the composition of transferrins indicate that they consist of two similar polypeptide halves, each of which contains one iron binding site (and in HST, one oligosaccharide each). Since the physical evidence indicates only one polypeptide chain per molecule, the apparent duplication in composition is probably not due to subunits. It might, however, result from a gene duplication process. Such duplications, involving peptide segments of shorter lengths, have been reported. $\gamma$-Globulin light chains appear to have evolved from duplication of a ~12,000 mol wt polypeptide (Lennox and Cohn, 1967), and some haptoglobin $\alpha$ chains appear to be multiples of ~8000 mol wt peptide segments (Dixon, 1966). Perhaps the evolutionary antecedent of transferrins was ~38,000 mol wt with one $Fe^{3+}$.

A model with two globular regions joined by a peptide chain has been proposed for BSA (Harrington *et al.*, 1956; Foster, 1960; Adkins and Foster, 1965) on the basis of fluorescence polarization studies and pH-dependent expansion below pH 4. OT (Phelps and Cann, 1956) and HST (Roberts *et al.*, 1966) have also been reported to undergo pH-dependent conformation changes below pH 4.5 as evidenced by decrease in sedimentation coefficient, without decrease in molecular weight. Whether this reflects a gross similarity to BSA in structure is speculative. However, it might be convenient to consider a similar model for transferrin structure.

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# Isolation and Characterization of Rabbit Serum and Milk Transferrins. Evidence for Difference in Sialic Acid Content Only*

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ABSTRACT: Transferrin has been isolated from rabbit serum by diethylaminoethyl Sephadex chromatography and electrophoresis. A similar iron-binding protein has been isolated from rabbit milk whey by electrophoresis and gel filtration. The two proteins are readily crystallized from distilled water at pH 5.3. Measurements have been made of nitrogen and iron content, molecular weight, light absorption spectra in the visible and ultraviolet ranges, and amino acid composition. The proteins have also been compared by electrophoresis on cellulose acetate and starch gel, by double diffusion in agar against specific antisera, and by two-dimensional peptide mapping of tryptic digests. The two proteins appear identical by all of these methods of analysis except electrophoresis, but this difference is eliminated by treatment with neuraminidase. The changes in mobility of the proteins after treatment with neuraminidase suggest that in serum most transferrin molecules contain two sialic acid residues while a few have only one residue; however in milk most molecules probably have one sialic acid residue, with a small proportion of the molecules having two. The molecular weight of the protein observed in the ultracentrifuge and on gel filtration calculates to be 70,000 daltons. The number of peptides observed on the maps, when considered in conjunction with the amino acid composition, suggests that this protein consists of two subunits.

Rabbit milk whey is remarkable for its extremely high iron-binding capacity due to the presence of a high concentration of an iron-binding protein with similar electrophoretic mobility to that of rabbit serum transferrin (Jordan *et al.*, 1967). In other species which have been studied iron-binding proteins are present in the milk in only low concentration and in the case of humans and the cow the major milk iron-binding protein is chemically and immunologically distinct from serum transferrin (Johansson, 1958, 1960; Montreuil and Mullet, 1960; Groves, 1960; Blanc and Isliker, 1961; Gorden *et al.*, 1962; Derechin and Johnson,

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