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Subunits of Human Transferrin

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1. Evidence for a subunit structure has been obtained by physical and chemical studies of reduced and alkylated or performic acid oxidized human transferrin. The molecular weights of the subunits were estimated to be between 39 400 and 44 000 by sedimentation and diffusion and by the approach to sedimentation equilibrium method. The sedimentation coefficient was found to decrease from 5 S to 1.2 S after reduction and alkylation in 8 M urea.

2. Fingerprinting patterns of trypsin digested material showed 34–38 ninhydrin positive spots, that is, approximately half the number of peptides expected from the arginine and lysine content of native transferrin. An identical fingerprint developed by the chlorine-iodine reaction revealed an additional peptide from which no N-terminal amino acid could be obtained.

Since Holmberg and Laurell [1] and Schade and Caroline [2] discovered human transferrin, the metal combining β_1-globulin, it has been thoroughly characterized with respect to its physicochemical properties,[3–5] the nature of the prosthetic iron,[6–8] and the carbohydrate structure.[9] That transferrin is a serum protein of great importance in the binding and transport of iron is well established. However, there is wide variation in the molecular weights reported for human transferrin. From iron-binding studies Jandl and Katz [10] calculated the molecular weight to 86 000. From sedimentation and diffusion constants Hazen, Jr.[11] reported a molecular weight of 82 500. Extreme values of 68 000 (Ref. 12) and 95 000 (Ref. 13) have been reported. From determinations of sedimentation equilibrium, sedimentation and diffusion, osmotic pressure and maximum iron-binding capacity, Roberts *et al.*[14] have recently reported molecular weights between 73 200 and 76 000 for four different preparations.

We have earlier reported a radical change in the sedimentation coefficient of native human transferrin after reduction and alkylation in 8 M urea.[15] These observations have now been extended by determining the molecular weights of the preparations by approach to sedimentation equilibrium, sedimentation and diffusion. The molecular weights determined for human transferrin after reduction and alkylation were in the range of 39 400—44 000,

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indicating the existence of two polypeptide chains. This was further supported by the number of tryptic peptides separated by fingerprinting: 38 instead of the theoretical 98.

MATERIALS AND METHODS

Purification of transferrin. Transferrin CC was isolated from fresh plasma by chromatography on DEAE-Sephadex and recycling chromatography on Sephadex G-200 according to a method described elsewhere.[16] The preparation was pure by the criteria of immunoelectrophoresis, polyacrylamide gel electrophoresis, and absorption spectra.

Reduction and alkylation in 8 M urea. The procedure was performed essentially according to Crestfield, Moore and Stein.[17] 25 mg iron-free transferrin was mixed with 3.61 g of deionized, crystalline urea [11] in a 10 ml vial. 300 μl of a 5 % EDTA solution, 3.0 ml of 1.5 M Tris-HCl buffer, pH 8.6 (Sigma 121), and 100 μl of β-mercaptoethanol (Eastman Organic Chemicals) were added. The volume was brought to 7.5 ml with water. The vessel was flushed with nitrogen and kept closed in darkness during the whole reaction. After 4 h at room temperature (20—22°) a freshly prepared solution of 268 mg recrystallized iodoacetic acid in 1.4 ml 1.0 M NaOH was added in darkness and allowed to react for 15 min. The reaction mixture was then dialyzed against cold water for 48 h, during which time the alkylated material precipitated. The precipitate was washed in cold water and dried *in vacuo*.

Starch gel electrophoresis. Electrophoresis was performed in urea-formate [19] gel at pH 3 or in urea-glycine [20] gel at pH 7 to 8. Samples (0.4—0.6 mg) were dissolved in 20 μl urea-formate buffer or urea-glycine buffer and transferred to small strips of Whatman 3 MM paper which were then inserted into slots in the gels. Electrophoresis was run horizontally at room temperature in an LKB-apparatus for 16 h. The urea-glycine gels were run at 250 V, 16 mA, and urea-formate gels at 125 V, 14 mA. After electrophoresis the gels were sliced and stained with Amido Black B.

Gel chromatography. A chromatography column was calibrated as described by Andrews [21] using Sephadex G-200 in 0.1 M Tris-HCl, pH 8.6. The column dimensions were 1.15 × 130 cm, from which the total bed volume was calculated to be 135 ml. The column was calibrated by determining elution volumes for 6 isotopically labelled proteins. Human γG-globulin (MW 160 000) (Ref. 19), ovalbumin (MW 46 000) (Ref. 22), pepsin (MW 35 000) (Ref. 23), and RNAse (MW 13 700) Ref. 24) were labelled with ^{125}I according to McFarlane.[25] Elution volumes were also determined for ^{131}I-labelled human serum albumin (RISA, Amersham, England) and ^{59}Fe-transferrin. Blue Dextran (Pharmacia, Uppsala) was used to determine the void volume = 38 ml. Three labelled protein preparations could be chromatographed simultaneously with reduced and alkylated transferrin, the latter being registered continuously at 280 mμ.[26] The column was eluted at room temperature at a flow rate of 1.5 ml/h with 0.1 M Tris-HCl buffer, pH 8.8, containing merthiolate (1:10 000) as preservative. 200 μl of each 30 min fraction was transferred to 10 ml of Bray's scintillation fluid [27] and counted in a Packard Tri Carb Model 3003 liquid scintillation counter.

Ultracentrifugation. Ultracentrifugation studies were performed in a Spinco Model-E ultracentrifuge equipped with phase plate schlieren optics. Sedimentation velocity experiments were performed at 59 780 rpm at 20° in 12 mm 4° sector cells with standard and wedge windows or in synthetic boundary cells. The diffusion coefficients were estimated by a method of Fujita-van Holde.[28] The diffusing boundary was measured in the capillary type, double sector, synthetic boundary cell at a speed of 4908 rpm. The observed sedimentation and diffusion values were corrected to the viscosity and density of water in the usual manner,[29] to obtain the $S_{20,w}$ and $D_{20,w}$ values. Sedimentation and diffusion coefficients were determined at four concentrations each and extrapolated to infinite dilution. Molecular weights were determined for individual protein concentrations by approach-to-equilibrium experiments, which were evaluated by two methods, those of Ehrenberg [30] and Trautman.[31] The partial specific volume was assumed to be 0.725 cm^3/g, (Refs. 32, 33).

Fingerprinting. Trypsin digestion was performed on fully reduced and iodoacetic acid alkylated transferrin and also on performic acid oxidized transferrin. 20 mg of

native transferrin was dissolved in 1.0 ml 99 % formic acid and 0.3 ml methanol was added with stirring. Performic acid was prepared, according to Hirs [34], by adding 0.5 ml of 30 % hydrogen peroxide to 9.5 ml of 99 % formic acid and the solution was allowed to stand at room temperature for 2 h. The protein solution and 2 ml of performic acid were cooled separately to −10° and then mixed. After a reaction time of 2.5 h the mixture was diluted with 10 volumes of ice water, dialyzed against cold water for 24 h and freeze dried.

A 0.2 % protein solution in 0.01 M NH_4OH at a final pH of 8.4 was digested with trypsin for 5 h at 37° at a 1:50 enzyme:substrate ratio.[35] Electrophoresis of 2.0 mg digested sample was run in pyridine:acetic acid:water, (100:10:890), pH 6.1, on Whatman 3 MC paper, 46 cm × 57 cm. Duplicate samples were run on the same paper on a water-cooled plate at 1.5 kV for 5 h. Each half was then sewn to a new Whatman paper. Chromatography was performed in a second dimension in pyridine:isoamyl alcohol:water (35:35:30, by vol.) [36] for 20 h at 24°. One of the fingerprints was developed by dipping in 0.2 % ninhydrin in acetone and drying at room temperature. The other fingerprint was transferred to a glass tank containing a chlorine atmosphere which was produced by mixing equal parts of 10 % HCl (w/v) and $KMnO_4$ (3.16 g/l). After 15—20 min exposure to chlorine, the chromatogram was hung in a hood and fanned overnight. Peptide spots were developed by spraying with a mixture of equal parts of 2 % potassium iodide and 2 % freshly prepared starch solution.[37] The developed spots were stable in darkness for several months.

Isolation of peptides. Fingerprinting revealed a peptide which failed to react with ninhydrin but which gave a positive chlorine-iodine reaction. This, along with an adjacent peptide, was isolated as follows. 80 mg of performic acid oxidized and trypsin digested transferrin was applied to a 30 cm long line on Whatman 3 MC paper. Electrophoresis was run at 1.5 kV for 6 h in the pyridine:acetic acid:water system, pH 6.1. A guide strip was developed with ninhydrin and the desired peptide material was eluted with 1 M acetic acid. The eluates were freeze dried, dissolved in 100 µl of 1 M acetic acid and applied to a 20 cm long line on a new Whatman 3 MC paper. Chromatography was then carried out in the pyridine:isoamyl alcohol:water system. Two guide strips were developed, one with ninhydrin and the other with the chlorine-iodine reaction. The desired peptides were eluted with 1 M acetic acid.

Amino acid analyses. N-Terminal amino acids were determined on reduced and alkylated transferrin according to Eriksson and Sjöquist [38] with a few modifications.[35] The protein was moistened overnight in 1.0 ml water and the pyridine-triethylamine buffer, adjusted to pH 10.0, was then added. The N-terminal amino acids were released as phenylthiohydantoin derivatives at 40° for 2.5 h in a solution made up of 1.0 ml water and 2.0 ml of hydrogen chloride saturated acetic acid. The sample was then taken almost to dryness by evaporation *in vacuo*. Water was added and the thiohydantoins were extracted with 3 volumes of ethyl acetate. Identification and quantitation of the thiohydantoins were performed by thin layer [39] and paper chromatography.[40]

The N-terminal amino acids of the isolated peptides were also identified as phenylthiohydantoin derivatives.[40] 100 µl of a solution containing 2.0 ml 2.0 M acetic acid, 1.2 ml distilled triethylamine, and distilled water to 25 ml was added to 0.1—0.15 µmole of a peptide and mixed with 100 µl of a solution containing 30 µl distilled phenylisothiocyanate in 5 ml acetone. After PTC-coupling for 3 h at room temperature, the samples were dried *in vacuo* over P_2O_5 in a desiccator for 10—15 h. Cyclization, extraction, and identfiication of the phenylthiohydantoins were performed as described for reduced and alkylated transferrin.

For amino acid analysis isolated peptides were hydrolyzed in 6 N HCl at 110° for 22 h in tubes flushed with N_2. The amino acids were converted to phenylthiohydantoin derivatives and determined by paper chromatography.

RESULTS

Investigation of the solubility properties of reduced and carboxymethylated transferrin showed that it could be dissolved in 0.1 M Tris-HCl, pH 8—9. To avoid nonspecific carboxymethylation the pH was kept above 8.3. Desalting

on Sephadex G-25, equilibrated with 0.1 M Tris-HCl, pH 8.6, gave a polydisperse product as shown by sedimentation velocity experiments. The procedure given in Materials and Methods, by which small molecules were removed by dialysis against water, was therefore preferable for preparing reduced and alkylated transferrin.

Starch gel electrophoresis of reduced and alkylated transferrin in 8 M urea-glycine buffer, pH 7—8, showed a single band even after extended running time. The same type of electrophoresis, but in 8 M urea-formate buffer, pH 3.9, gave a major band and two minor ones with lower mobility. The latter represented less than 10 % of the total material and corresponded to a fraction eluted with the void volume in chromatography on Sephadex G-200 (Ref. 15). The elution pattern of the minor bands and their behaviour in starch gel electrophoresis suggests that they probably consisted of aggregated material. The change in sedimentation coefficient accompanying reduction and alkylation of transferrin suggested the existence of subunits. Attempts were made to separate these. Reduced and alkylated material was chromatographed on CM-cellulose (Brown and Co., Berlin, N. H., U.S.A.) equilibrated with 0.03 M phosphate buffer, pH 4.5, containing 8 M deionized, recrystallized urea. The first protein peak was eluted with 0.03 M phosphate buffer, pH 4.5, 8 M in urea. Gradient elution was then started with 0.1 M phosphate buffer, pH 6.0, 8 M in urea. The second peak to emerge contained the same amount of material as the first. These two fractions had the same mobility on starch gel electrophoresis both in urea-glycine and in urea-formate buffers. N-Terminal amino acid determinations showed that the main N-terminal, valine, occurred in the same amount in both fractions. The two peaks obtained were presumed to be the two fractions of normal transferrin (transferrin I and II) observed earlier.[41] Subunits were not separated.

We have recently reported that normal human transferrin contained 0.1—0.2 moles/mole of N-terminal aspartic acid, glycine, and serine in addition to 0.8—0.9 moles of valine/mole.[35] N-Terminal determinations on reduced and alkylated transferrin yielded 0.9 mole valine/mole and 0.3 moles of each of the additional amino acids. The calculations were based on an assumed molecular weight of 88 000 for transferrin. The amount of N-terminal aspartic acid, glycine, and serine in reduced and alkylated transferrin was 2—3 times that found in native material. The extraneous N-terminal amino acids may have been due to contamination, to partial hydrolysis of susceptible peptide bonds during preparation, or to the existence of several subunits of transferrin. It has not been possible to establish the origin of these minor N-terminal amino acids, which increased in amount after reduction and alkylation.

Ultracentrifugation studies. Sedimentation velocity studies were performed with reduced and alkylated transferrin which was either desalted by dialysis against distilled water and lyophilized or desalted by gel filtration on Sephadex G-25. In the first case a homogeneous peak was obtained (Fig. 1a) with a $S_{20,w}$ value of 1.18 S at a protein concentration of 0.42 %. Ultracentrifugation of the same material at protein concentrations of 0.05, 0.18, and 0.26 % gave similar sedimentation coefficients. The result of this study, shown in Fig. 2, gave a $S^{\circ}_{20,w}$ value of 1.24 S when extrapolated to zero concentration. In contrast, reduced and alkylated transferrin which was desalted on Sephadex

Fig. 1. Schlieren patterns of sedimentation velocity experiments on reduced and alkylated transferrin in a synthetic boundary cell at a rotor speed of 59 780 rpm, 20°, in 0.1 M Tris-HCl buffer, pH 8.6. a) dialyzed and lyophilized material, protein concentration 4.2 mg/ml, bar angle 50°; time, 72 min; $S_{20,w}$ = 1.18 S. b) gel filtered material, protein concentration 4.0 mg/ml, bar angle 50°, time, 32 min, $S_{20,w}$ = 6.35 S and 2.40 S.

G-25 gave a heterogeneous sedimentation pattern. At least two peaks appeared, representing $S_{20,w}$ values of 6.35 S and 2.40 S, respectively (Fig. 1b). The sedimentation coefficients of different preparations varied. Molecular weight determinations on homogeneous material (dialyzed and lyophilized) by the approach-to-equilibrium method yielded values in the range of 42 000—44 000.

Fig. 3 shows the diffusion coefficients determined at four different protein concentrations. The reduced and alkylated transferrin preparations were dialyzed extensively against 0.1 M Tris—HCl, pH 8.6, for 72 h. The $D°_{20,w}$ value was calculated to be 2.8 × 10^{-7} cm²/sec. This value, when substituted into the Svedberg equation along with the $S°_{20,w}$ value, gave a molecular weight of 39 400.

Molecular weights of the transferrin subunits were also determined at constant protein concentrations by use of the Archibald principle. Small

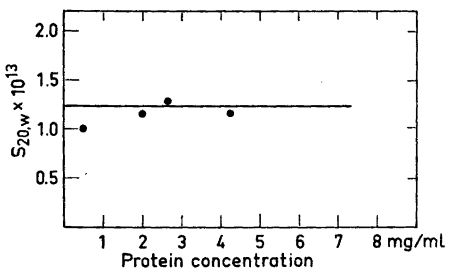

Fig. 2. Plots of sedimentation coefficients against protein concentration. All measurements were carried out in 0.1 M Tris-HCl, pH 8.6, $S°_{20,w}$ = 1.24 S. Less weight was given to the S-value at the lowest concentration in the extrapolation due to experimental uncertainties.

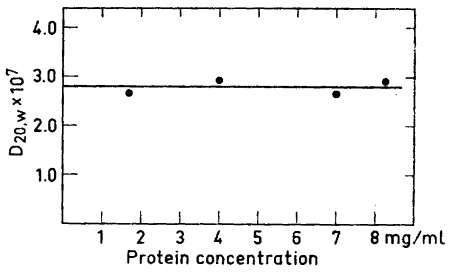

Fig. 3. Plots of diffusion coefficients against protein concentration. All measurements were carried out in 0.1 M Tris-HCl, pH 8.6, $D°_{20,w}$ = 2.80 × 10^{-7} cm²/sec.

variations were obtained, 41 500 by the method of Trautman [31] and 41 000—44 000 by the method of Ehrenberg.[30] The two methods were applied to different concentrations of protein. The values calculated according to Ehrenberg represent four determinations performed with the reduced and alkylated transferrin kept in solution at 4° for 1 to 520 h. The Trautman value given is an average of two different determinations.

Gel chromatography studies. Gel chromatography of reduced and alkylated transferrin on Sephadex G-200 in 0.1 M Tris-HCl, pH 8.6, showed a symmetrical peak preceding native transferrin. The elution volumes of this and native transferrin were 41 and 66 ml, respectively.

Radioactively labelled γG-globulin, native transferrin, albumin, ovalbumin, pepsin, and ribonuclease were chromatographed along with reduced and alkylated transferrin on the Sephadex G-200 column. The elution volume was then plotted against the logarithm of the molecular weight for each protein. The curve was consistent with the theory of Andrews,[21] who stated that the elution volumes of most globular proteins are directly proportional to the logarithms of their molecular weights. However, when the molecular weight of reduced and alkylated transferrin was calculated from the elution volume by means of the graph, an apparent molecular weight more than twice that of native transferrin was obtained.

Laurent and Killander [42] have shown that the separation by gel chromatography of molecules varying in size can be explained as a steric exclusion of solutes from the gel phase. Their parameter K_{av}, which is the fraction of the

Table 1. Evaluated radii and $K^a{}_v$ values for native and for reduced and alkylated transferrin.

Transferrin	$D^\circ_{20,w} \times 10^7$	$r_s \times 10^8$	Ve (ml)	K_{av}
Native	5.85[a]	36.7	66	0.33
Reduced and alkylated	2.80	76.7	41	0.04

[a] Taken from Roberts, R. G. *et al.*[14]

gel phase volume available for the solute, was determined from the elution volume, the void volume, and the total volume of the column. The radius, r_s, of an equivalent sphere was calculated from the diffusion constants, using Stoke's formula.[43] The calculations give an estimation of the molecular size of a protein.

The K_{av} values of the test proteins including native transferrin were almost identical with those calculated by Laurent and Killander from the experiments of Killander. The K_{av} and r_s values calculated for native and for reduced and alkylated transferrin coincided almost exactly with the theoretical curve given for Sephadex G-200. Reduced and alkylated transferrin was found to have a larger molecular size than native transferrin; *i.e.* the Stoke's radius was 76.7×10^{-8} cm *versus* 36.7×10^{-8} cm for the native protein.

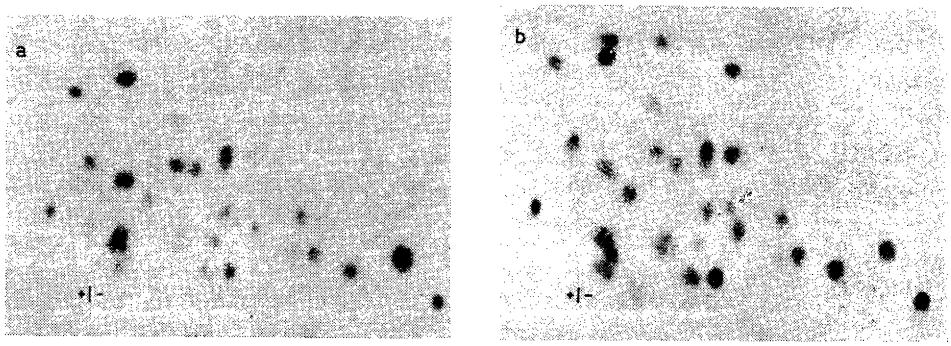

Fig. 4. Duplicate fingerprints of trypsin digested performic acid oxidized transferrin developed by a) the ninhydrin reaction and b) the chlorine-iodine reaction.

Fingerprinting. Fig. 4a shows a fingerprint of performic acid oxidized transferrin developed with ninhydrin. 34—38 strongly staining peptide spots could always be identified, *i.e.* less than half of the number predicted from the 30 arginines and 68 lysines present per mole of transferrin.[44] Transferrin also contains 22 histidine residues per mole. Specific staining [45] for arginine and histidine gave 10—14 and 12 positive spots, respectively. These results, together with the number of tryptic peptides, suggested that transferrin may consist of two polypeptide chains with similar primary structure.

A duplicate fingerprint, Fig. 4b, was developed by the chlorine-iodine reaction. One additional spot was obtained, indicated in the figure by an arrow. A positive chlorine-iodine reaction but a failure to react with ninhydrin suggested the presence of a blocked N-terminal amino acid in the indicated peptide. This peptide and a control peptide were isolated according to the procedure described in Materials and Methods. No phenylthiohydantoin derivative of an N-terminal amino acid could be recovered from the marked peptide, but the control that is located just beneath, yielded N-terminal valine. Amino acid analysis showed that the indicated peptide contained serine, phenylalanine, and arginine. The control peptide contained valine, asparagine or aspartic acid, proline, and arginine. The fingerprints of trypsin digested, reduced, and alkylated transferrin gave similar results.

DISCUSSION

Reduction and alkylation of human transferrin in 8 M urea-Tris-HCl buffer, pH 8.6, produced a radical change in the sedimentation coefficient compared with that of native transferrin. The material prepared by the dialyzing and freeze-drying technique was always homogeneous in sedimentation velocity experiments. The sedimentation coefficients varied but molecular weight determinations gave values corresponding to half the molecular weight of native transferrin. The results indicate that normal human transferrin consists of two subunits, each having a molecular weight of about 40 000.

These results imply that the molecular weights of native transferrin is closer to 80 000 than to 90 000. The large discrepancy in molecular weights reported in the literature (68 000—95 000) may be due to the fact that the preparations used contained impurities of higher molecular weight or to the use of different partial specific volumes. It is not known whether the subunits are held together by covalent bonds. Amino acid analysis of native transferrin showed 44 half-cystine residues/mole.[44] No free sulfhydryl groups could be detected.[33] Transferrin therefore must contain 22 disulfide bridges which may be interchain or intrachain. Preliminary ultracentrifugation studies showed that treatment with 8 M urea alone brought about a decrease in the sedimentation coefficient to 3 S. The molecular weight of this material was the same as that of native transferrin.

Transferrin has been assumed to consist of a single polypeptide chain. However, the molecule binds two molecules of iron and structural data from similar metal binding proteins suggest by analogy that transferrin may consist of a pair (or pairs) of subunits. Jamieson [9] has isolated two branched glycopeptides from human transferrin with a molecular weight of 2 350 each. They are identical with respect to carbohydrate content and together contain 8 moles of N-acetylglucosamine, 4 moles of galactose, 8 moles of mannose, and 4 terminal sialic acids per 90 000 g glycoprotein. The finding of two identical heterosaccharide chains supports the theory of a single glycopeptide for each subunit. The presence of two polypeptide chains was also suggested by the fact that fingerprints of reduced and alkylated or performic acid oxidized transferrin contained only half the expected number of tryptic peptides. Furthermore, specific staining for arginine and histidine showed about one half the number of positive peptides predicted by amino acid analyses. These results also suggest that the primary structure of the subunits must be similar except for their N-terminal amino acids. Transferrin appears to contain only one free N-terminal amino acid, valine, per mole if one ignores the small amounts of additional amino acids. Ninhydrin failed to react with a tryptic peptide spot which was positive in the chlorine-iodine reaction. This suggests the presence of a blocked N-terminal amino acid in one of the subunits if that peptide is one of the N-terminal peptides. Such a block may be due to the presence of an acyl- or a pyroglutamic acid residue or a cystine. Since preliminary amino acid analyses of the peptide, recovered from performic acid oxidized transferrin, were negative for glutamic acid and cysteic acid, pyroglutamic acid and cystine can be excluded as N-terminal amino acids.

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