Comparison of the Sedimentation and Gel-Filtration Behaviour of Human Apotransferrin and its Copper and Iron Complexes

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Equilibrium-dialysis experiments showed that Tris or citrate in the solution prevented copper from occupying completely the specific metal-binding sites on human transferrin. Differential measurements of sedimentation velocity under conditions where two atoms of copper per molecule of protein were bound showed an increase in $s_{20,w}^0$, relative to that of the apoprotein, practically the same as that produced by two atoms of iron. Gel-filtration experiments made under the same conditions to investigate the effect of copper binding on the Stokes radius of the protein showed merely that it lost most of the metal as it passed down the column.

The role of transferrin in sequestering and transporting iron in the body has prompted many investigations, both biological and physical. In particular, gel-filtration experiments with human transferrin indicated that the protein emerged slightly later when complexed with iron (Kornfeld, 1969). Similar experiments with radioactively labelled transferrin and differential sedimentation-velocity measurements (Charlwood, 1971) demonstrated that specific binding of two atoms of iron per molecule produced a decrease in Stokes radius of $1.4 \pm 0.17\%$ (s.d., nine experiments) and an increase in $s_{20,w}^0$ of $1.8 \pm 0.36\%$ (s.d., 13 experiments). In view of slight uncertainties in partial specific volumes these values are compatible with a change in molecular conformation without the necessity of postulating a change in average molecular weight that would arise if there were rapid reversible association more favoured by one species than by the other.

The work described below aimed to answer the following questions. Does substitution of K^+ for Na^+ ions in the buffer system, or decreasing the salt strength to a value near iso-osmotic, have any influence on the hydrodynamic consequences of iron binding? Does copper, which binds at the same specific sites as iron, produce comparable effects? To answer the last question, it proved necessary to study the influence of conditions on the binding of copper to the protein. This was done by equilibrium-dialysis experiments that involved radioactive copper.

Materials and Methods

Preparation of transferrin

Batches 1 and 3 from the previous work were used (Charlwood, 1971), as well as a new batch (4) that was made as follows. A 0.4% solution of rivanol (6,9-diamino-2-ethoxyacridine lactate), obtained from

Koch-Light Laboratories, Colnbrook, Bucks., U.K., was prepared in sodium phosphate buffer, pH 8.0, I 0.1. The precipitate that formed when 350 ml of this solution was added slowly to 100 ml of stirred human plasma was separated by low-speed centrifugation. The supernatant, after dialysis against 0.1 M-NaCl–17 mM-Tris–HCl buffer, pH 7.2, and clarification by further low-speed centrifugation, was divided into three equal parts. These were passed in turn down a column (2.2 cm $\times$ 50 cm) of Sephadex G-25 (Pharmacia Fine Chemicals, Uppsala, Sweden) prepared in the same solvent. The combined protein effluent, free of rivanol, was concentrated to 30 ml by dialysis under pressure (Thorup, 1966) against 0.3 M-NaCl–50 mM-Tris–HCl buffer, pH 7.2. This solvent was also used to pack a column (3.5 cm $\times$ 140 cm) of Sephadex G-150, for the next stage. The protein in the last peak that emerged was concentrated to 20 ml by dialysis under pressure as above against 0.12 M-Tris–HCl buffer, pH 8.0, and applied to a column (1.5 cm $\times$ 90 cm) of DEAE-Sephadex A-50 washed with this buffer. Elution at a flow rate of 14 ml/h was carried out with a gradient from a device (Hegenauer *et al.*, 1965) that originally had 85 ml of the 0.12 M-Tris–HCl buffer in its inner compartment and 160 ml of 0.2 M-Tris–HCl buffer, pH 8.0, in the outer. Elution was completed with the 0.2 M-Tris–HCl buffer and the material in the second main peak was pooled and concentrated by dialysis under pressure.

Sedimentation and gel filtration

These techniques and associated calculations were as previously described (Charlwood, 1971) with one exception. Because of the likelihood of partial decomposition if cupric chloride were dried down completely, solutions for differential sedimentation-velocity work were prepared as follows. A solution

of about 25 mg of the apoprotein/ml, the concentration of which had been measured accurately in a differential refractometer (Cecil & Ogston, 1951), was divided into equal parts. From accurate micro-syringes (Scientific Products Inc., Flushing, N.Y., U.S.A.) these samples received a small volume of water or an equal volume of cupric chloride solution respectively. The copper added was calculated to provide 2.2 atoms/molecule of protein, based on a nominal molecular weight of 80000 and specific refractive increment of 0.0018. Accurate dilutions of the two protein solutions provided a set of samples that were identical in pairs save for the presence or absence of copper.

Equilibrium dialysis

This was done in simple cells that consisted of two Perspex blocks clamped tightly together with nuts and bolts. The blocks, each of which contained a cylindrical cavity (1.9 cm diameter $\times$ 0.48 cm), were separated by a membrane cut from dialysis tubing that had been treated to remove any iron present (Charlwood, 1971). The compartments were filled by syringes through narrow holes in the blocks which were subsequently sealed by nylon set-screws. The cells were rocked in closed boxes at 4°C with the membranes perpendicular to the axis of the rocker. Checks were made to confirm that evaporation from the cells was negligible during the time required for the attainment of dialysis equilibrium (2–3 days). Mixtures were made by means of the micro-syringes, so that generally cells could be set up in pairs that were identical in their total contents, but differed initially according to whether or not the copper was in the protein compartment.

Radioactive copper

The ^{64}Cu was obtained as cupric chloride from The Radiochemical Centre, Amersham, Bucks., U.K. It was either used as such or converted into the citrate complex by mixing with a small volume of sodium citrate solution. Radioactivity was measured, with automatic background subtraction, in a Packard Autogamma counter. The 12.8 h half-life of the isotope restricted the number of successive experiments possible with one batch, but adequate count rates were obtainable when dialysis equilibrium was practically attained.

Results

Iron complexes

Sedimentation coefficients determined in 0.3 M-KCl–50 mM-Tris–HCl buffer, pH 8.0, are shown in Fig. 1. The coefficient of linear dependence on concentration, k , given by the slopes of the regression

lines is 0.090 and 0.089 for apotransferrin and iron-saturated transferrin respectively, values that are not significantly different from each other or from the equivalent results obtained in 0.3 M-NaCl–50 mM-Tris–HCl buffer, pH 8.0 (Charlwood, 1971). The difference in $s_{20,w}^0$ for the two forms of the protein, $0.079 \pm 0.035 \text{ S}$ (s.d., 10 experiments) was also virtually the same, but not the most sensitive way of comparing their sedimentation behaviour. The difference δs^{c_0} was not significantly dependent on c_0 (Fig. 2) where c_0 is the initial protein concentration, the best estimate in these circumstances being the simple mean, $0.079 \pm 0.017 \text{ S}$ (s.d., 10 experiments). Since, within experimental error, this is the same as the corresponding value obtained earlier (Charlwood, 1971) in the NaCl-based solvent, the values for both

Fig. 1. $s_{20,w}$ values as a function of concentration (c_0) for apotransferrin ($\square$, Δ) and iron-saturated transferrin ($\blacksquare$, $\blacktriangle$) in 0.3 M-KCl–50 mM-Tris–HCl buffer, pH 8.0 Δ and $\blacktriangle$, Batch 1; $\square$ and $\blacksquare$, batch 3.

Fig. 2. Differences of $s_{20,w}$ for apotransferrin and iron-saturated transferrin as a function of concentration in 0.3 M-KCl–50 mM-Tris–HCl buffer, pH 8.0

Open symbols indicate experiments in which the apo-protein was contained in the cell with a wedge window: Δ and $\blacktriangle$, batch 1; $\square$ and $\blacksquare$, batch 3.

sets of conditions were combined, giving $0.086 \pm 0.017S$ (s.d.). The results of sedimentation experiments in 0.1 M-NaCl–50 mM-Tris–HCl buffer, pH 8.0, were too erratic to allow a similar analysis to be made meaningfully.

The gel-filtration findings are summarized in Table 1, the first line of which is based on the earlier work (Charlwood, 1971). The exceptionally small standard deviation for the experiments with iron-saturated transferrin in the last line is presumed to have arisen by coincidence, accentuated by the small number of observations. Further statistical analysis of the results showed that (a) substitution of K^+ for Na^+ ions at the same concentration had no effect, (b) in a given solvent the effect of binding two atoms of iron is indistinguishable from double the effect produced by one and (c) decreasing the concentration of salt present caused an apparent decrease in the magnitude of the effects.

Copper complexes

The results of dialysis experiments in the presence of Tris buffer are given in Table 2. Although insufficient time was allowed for equilibrium to be reached in the cells to which the copper was added as chloride, it is clear that in none of the cells did the binding of copper to protein approach two atoms/molecule, although it did rise, to a diminishing degree, as the total copper concentration was increased. The last two lines of Table 2 prove that this must have been due to competition from the Tris, even though citrate may have played some part. When Tris buffer was replaced by phosphate buffer, the results shown in Table 3 were obtained. They demonstrate that, even at very low concentrations, citrate competes quite effectively with the protein for the available copper. Only when copper was added as the chloride, in the complete absence of citrate, did the copper bind practically quantitatively to the transferrin.

Table 1. *Gel-filtration differences between apotransferrin and iron-transferrins*

Results are means $\pm$ s.d. for the quoted numbers of experiments.

Solvent	No. of experiments	Difference between Stokes radius (nm) of apotransferrin and	
		(a) 1-Fe-atom form	(b) Fe-saturated form
0.3 M-NaCl–50 mM-Tris–HCl, pH 8.0	9	$0.023 \pm 0.005_1$	
	8		$0.049 \pm 0.005_3$
0.3 M-KCl–50 mM-Tris–HCl, pH 8.0	5	$0.024 \pm 0.002_5$	
	5		$0.043 \pm 0.005_9$
0.1 M-NaCl–50 mM-Tris–HCl, pH 8.0	3	$0.016 \pm 0.003_1$	
	3		$0.028 \pm 0.000_3$

Table 2. *Equilibrium dialysis of copper and human transferrin (5.3 mg/ml) in 0.3 M-NaCl–10 mM-NaHCO₃–50 mM-Tris–HCl buffer, pH 8.0*

For experimental details see the Materials and Methods section.

Copper added (atoms/molecule of protein)	Copper bound (atoms/molecule of protein)	Compartment receiving copper	Dialysis time (h)
2.0	0.618	Protein	72
	0.591	Buffer	72
3.0	0.724	Protein	72
	0.777	Buffer	72
4.0	0.926	Protein	72
	0.903	Buffer	72
6.0	1.096	Protein	72
	1.072	Buffer	72
4.5*	1.316	Protein	24
	1.167	Buffer	24

* Copper was added as chloride to these cells, but as citrate to the rest with copper and citrate in equimolar amounts.

Table 3. *Equilibrium dialysis of copper and human transferrin in 0.3 M-NaCl-10 mM-NaHCO₃-sodium phosphate (I 0.05) buffer, pH8.0*

For the experimental details see the Materials and Methods section.

Molar ratio of citrate/copper	Protein concentration (mg/ml)	Copper added (atoms/molecule of protein)	Copper bound (atoms/molecule of protein)	Compartment receiving copper	Dialysis time (h)
1.5	4.6	2.1	1.215	Protein	72
1.5	4.6	2.1	1.204	Buffer	72
1.5	4.6	2.4	1.254	Protein	72
1.5	4.6	2.4	1.294	Buffer	72
1.5	4.6	3.0	1.404	Protein	72
1.6	4.6	2.1	1.166	Protein	48
1.6	4.6	2.1	1.165	Buffer	48
1.6	4.6	2.4	1.250	Protein	72
1.6	4.6	2.4	1.222	Buffer	72
1.6	4.6	3.0	1.358	Protein	72
1.6	4.6	3.0	1.346	Buffer	72
1.5	9.3	10.8	1.695	Protein	120
1.5	9.3	10.8	1.668	Buffer	120
1.5	15.2	0.56	0.522	Protein	72
1.5	15.2	0.56	0.520	Buffer	72
1.5	15.2	0.87	0.817	Protein	72
1.5	15.2	0.87	0.803	Buffer	72
1.5	15.2	1.4	1.096	Protein	72
1.5	15.2	1.4	1.099	Buffer	72
1.5	15.2	2.1	1.251	Protein	72
1.5	15.2	2.1	1.271	Buffer	72
1.5	15.2	3.4	1.402	Protein	72
1.5	15.2	3.4	1.301	Buffer	72
*	15.0	2.32	2.228	Protein	72
*	15.0	2.12	2.013	Buffer	72

* Copper was added as the chloride.

Fig. 3. *s_{20,w} values as a function of concentration for apotransferrin (Δ, ○) and copper-saturated transferrin (▲, ●) in 0.3 M-NaCl-10 mM-NaHCO₃-sodium phosphate (I 0.05) buffer, pH8.0*

Δ and ▲, Batch 1; ○ and ●, batch 4.

Fig. 4. Differences of $s_{20,w}$ for apotransferrin and copper-saturated transferrin as a function of concentration in 0.3 M-NaCl-10 mM-NaHCO₃-sodium phosphate (I 0.05) buffer, pH 8.0

Open symbols indicate experiments in which the apoprotein was contained in the cell with a wedge window: Δ and $\blacktriangle$, batch 1; $\circ$ and $\bullet$, batch 4.

Differential sedimentation measurements with apotransferrin and its copper complex were made under conditions governed by the above findings, producing the results shown in Figs. 3 and 4. The k values were 0.090 and 0.086 for apotransferrin and the copper complex respectively, and the difference in $s_{20,w}^0$ was 0.063 ± 0.014 S (s.d., 13 experiments). As there was again no detectable dependence of δs^0 on concentration, its mean was calculated. The value, 0.077 ± 0.012 S (s.d., 13 experiments), did not differ statistically from the corresponding value for iron-transferrin.

When a gel-filtration experiment was carried out with transferrin that had two atoms of copper/molecule attached, a peak of radioactivity emerged about one fraction later than that of the extinction at 280 nm. This was despite the use of the solvent defined in Table 2 and the addition of the copper as chloride. Moreover, only about 20% of the radioactivity applied to the column remained associated with the protein.

Discussion

Although it is evident that substitution of K⁺ for Na⁺ ions in the ionic environment of the protein does not alter the effects of iron binding, there is some doubt about the interpretation to be placed on the results of lowering the salt concentration. Whereas at higher ionic strength it was possible to measure both differential sedimentation-velocity and gel-filtration parameters, the erratic behaviour during sedimentation at the lower ionic strength made it impossible to confirm that the smaller effects on columns were due to a different change of Stokes radius. The lack of reproducibility in the sedimentation measurements, though significant in this context,

would quite likely have escaped notice in ordinary velocity measurements.

The successful competition by Tris for copper, demonstrated in Table 2, is understandable because of the moderate concentration of Tris used and the log K values of 3.98, 3.49 and 3.2 (where K is the appropriate binding constant) reported by Hall *et al.* (1962) for the successive steps in the reaction of Tris with Cu²⁺ ions. It was somewhat unexpected that the very small amounts of citrate present in the dialysis experiments (Tables 2 and 3) should have exerted as much influence as they did. Had prior estimates been attempted on the basis of the equilibrium constants measured by Warner & Weber (1953) for the complexing of citrate with copper, it might have been possible to anticipate this.

The citrate/copper ratio of 1.5 in Table 3 was chosen because experiments showed that if lower ratios were used copper was precipitated in the phosphate environment in the absence of protein. However, these tests were done at much higher concentrations of copper than those used for work with transferrin. As the use of citrate in sedimentation experiments was precluded, the copper had to be added as simple Cu²⁺ ion, a procedure that again involved questions about its possible precipitation as phosphate or its partial hydrolysis. The last experiment in Table 3 showed that when copper was introduced as chloride into the buffer compartment of the dialysis cell it was able to pass through the membrane and combine with the protein. The chemical form of that which remained unbound is not known, but quite likely it was in true equilibrium with the bound copper, as the gel-filtration experiment showed that the binding was too weak to prevent a large proportion of the copper from becoming detached during passage of the protein down the column.

It has long been known that copper is bound by transferrin much less firmly than iron is (see, e.g., Aasa *et al.*, 1963). This was apparent in the present work too and was the reason why the concentration of bicarbonate, which forms part of the yellow complex, was maintained at 0.01 M. It was thus rather interesting to discover that the effects of the two metals on the sedimentation coefficient of the protein were indistinguishable. Although different buffers were used with iron and copper respectively, the main salt contribution was similar. It was unfortunate that it was not possible to carry out satisfactory gel-filtration experiments to check that there were no complicating factors that would invalidate the simplest interpretation of the ultracentrifugation results obtained with copper, that they are a direct consequence of a change in conformation of the protein molecule.

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