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The competitive equilibrium between aluminium and ferric ions for the binding sites of transferrin

M. Cochran+, J. Coates* and S. Neoh++

Department of Medicine and Immunology, Flinders Medical Centre, Bedford Park, 5042 Australia, and *Department of Physical Chemistry, University of Adelaide, Adelaide, 5000 Australia*

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Human transferrin is shown to bind 2 mol of aluminium per mol of protein using spectrophotometric titration. Competitive equilibrium between aluminium and ferric ions for transferrin binding sites is observed, and a value of 2.5 ($\pm$ 0.4) $\times$ 10^{15} M^{-1} is found for the apparent binding constant under physiological conditions.

Human transferrin *Aluminium binding constant* *Molar relationship* *Aluminium–iron competition*

1. INTRODUCTION

Aluminium in the plasma of hemodialysis patients is largely protein-bound [1,2] and is now recognised to be associated with the transferrin fraction [3–5]. In view of the morbidity caused by aluminium in these patients, further characterization of the transferrin aluminium interaction is of obvious interest. Human transferrin has two Fe^{3+} binding sites, which, though not identical [6], are believed to be independent at pH 7.4 and very similar in their Fe^{3+} binding constants [7]. Aluminium, like iron [7], also requires bicarbonate for specific binding to occur [3]. In this study, UV spectroscopy shows that the transferrin molecule binds two Al^{3+}, and centrifugal ultrafiltration is used to demonstrate competition between Al^{3+} and Fe^{3+} for the binding sites. This permits calculation of values for the apparent transferrin–Al^{3+} binding constant, invoking the published value for the apparent transferrin–Fe^{3+} binding constant [7]. Because Fe^{3+} and Al^{3+} tend to hydrolyze at pH 7.4, all measurements were made in the presence of excess citrate. Thus analysis of the results must take into account the various ion species arising both from hydrolysis of the citrate complexes [6] as well as from the aquospecies themselves [8].

Abbreviations: OD_{240} optical density at 240 nm.

2. EXPERIMENTAL

2.1. *Protein and buffers*

Human transferrin (Sigma, St. Louis, MO) was dissolved in 10 mM EDTA/100 mM NaCl, pH 5.5, and filtered through an Amicon YM5 membrane, followed by three washes with buffer A. The final concentration was measured by immunodiffusion (Partigen, Behring). Plastic containers were used routinely, pre-cleaned with 10 mM EDTA followed by three washes in twice-distilled water. All experiments took place at 25°C.

Buffer A: 50 mM Tris HCl/100 mM NaCl/10 mM $NaHCO_3$, pH 7.4.

Buffer B: 25 mM sodium hydrogen citrate, pH 7.4.

2.2. *Spectrophotometric titration*

The OD spectrum of transferrin (17 μM) in buffer A was compared with a similar sample containing 80 μM $AlK(SO_4)_2$ in a Pye Unicam SP8/100 scanning spectrophotometer. The change in OD_{240} was then observed following titration of 1.0 mM

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$AlK(SO_4)_2$ in 10 μl portions to 3 ml of the transferrin solution in the cuvette. Distilled water was added to the reference cuvette at the same rate. Titration proceeded up to a 5-fold Al^{3+}/transferrin molar ratio.

2.3. *Aluminium–iron competitive binding*

100 μl portions of human transferrin (63 μM) in buffer A were equilibrated in a series of tubes with equal volumes of solutions of $AlK(SO_4)_2$ in buffer A, giving a range of concentrations from 0 to 200 nmol Al^{3+} per nmol transferrin. Ferric citrate, labelled with $^{59}Fe^{3+}$ (Amersham International, Amersham, England), was prepared in buffer B and 100 μl portions added to each tube to give 1.0 nmol Fe^{3+} per nmol transferrin. The mixture was shaken and left for 30 min before being centrifuged on Amicon Centrifree membranes at 2000 × *g* in a PR 6000 centrifuge (IEC, Needham, MA). Pilot experiments over 12 h periods had shown that equilibration had occurred within 30 min. The $^{59}Fe^{3+}$ in the ultrafiltrate was measured, and the proportion bound to transferrin calculated. The retention of $^{59}Fe^{3+}$ by the membrane was examined using transferrin-free solutions.

2.4. *Competitive binding of Fe^{3+} and Al^{3+} to transferrin*

Consider the two sites of transferrin to be independent and identical, where [P]° and [P] = total and free transferrin site concentrations, respectively. For Fe^{3+} and Al^{3+} binding competitively, [P]° = [PFe^{3+}] + [PAl^{3+}] + [P]:

$$K'_{Fe} = \frac{[PFe^{3+}]}{([P]°-[PFe^{3+}]-[PAl^{3+}])([Fe^{3+}])} \quad \text{(i)}$$

$$K'_{Al} = \frac{[PAl^{3+}]}{([P]°-[PFe^{3+}]-[PAl^{3+}])([Al^{3+}])} \quad \text{(ii)}$$

Therefore:

$$K'_{Fe}[Fe^{3+}] = \theta_{Fe}/(1-\theta_{Fe}-\theta_{Al}) \quad \text{(iii)}$$

and

$$K'_{Al}[Al^{3+}] = \theta_{Al}/(1-\theta_{Fe}-\theta_{Al}) \quad \text{(iv)}$$

where θ_{Fe} and θ_{Al} are the proportions of transferrin sites bound by iron and aluminium respectively. Combining Eqns (iii) and (iv), we obtain

$$K'_{Al} = \frac{K'_{Fe}([Fe^{3+}])\left(\frac{1}{\theta_{Fe}}-1\right)-1}{[Al^{3+}]} \quad (1)$$

where K'_{Al} and K'_{Fe} are the apparent binding constants of transferrin for Al^{3+} and Fe^{3+}, respectively, under the conditions of the experiment.

3. RESULTS AND DISCUSSION

3.1. *Spectrophotometric titration*

The UV spectra of transferrin at pH 7.4 showed a sharp increase in OD_{240} in the presence of excess Al^{3+} (fig.1), broadly agreeing with the observations of others at pH 8.0 [3] and pH 8.4 [9]. The effect on OD_{240} of increasing the Al^{3+}/transferrin molar ratio (fig.2) showed there was a change in slope at a molar ratio of unity, and a plateau developed at a molar ratio of 2, consistent with stoichiometric binding of Al^{3+} at 2 sites.

3.2. *Competitive binding of Al^{3+} and Fe^{3+} to transferrin*

A decrease in the uptake of Fe^{3+} by transferrin was observed as the total Al^{3+}/Fe^{3+} concentration ratio increased (fig.3, table 1), indicating competition for the same binding sites. In the absence of

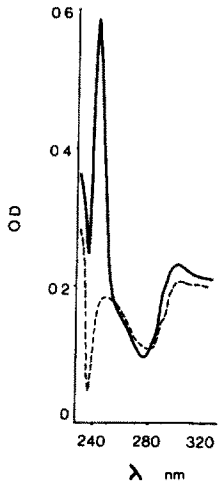

Fig. 1. The optical density spectra of apotransferrin (···) and the aluminium–transferrin complex (——) at pH 7.4 (buffer A).

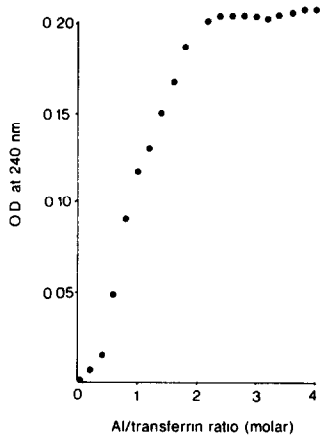

Fig. 2. The spectrophotometric titration at 240 nm of 17 μM transferrin with aluminium at pH 7.4 (buffer A).

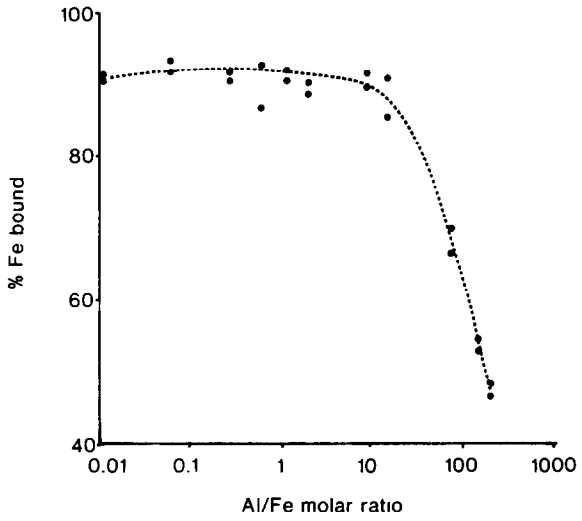

Fig. 3. The effect on Fe^{3+} binding to transferrin, of prior equilibration of the protein with Al^{3+} in a range of concentrations at pH 7.4 (buffer A + buffer B).

transferrin, 92–95% of the $^{59}Fe^{3+}$ could be recovered in the ultrafiltrate.

If it is assumed that at pH 7.4, transferrin's two binding sites are independent and have similar affinities for a particular metal, that all sites are available, and also that each binding event involves the incorporation of one HCO_3^-, then the apparent binding constant for transferrin and Al^{3+} may be expressed as in Eqn (1). $[Al^{3+}]$ and $[Fe^{3+}]$ are the equilibrium concentrations of free, uncomplexed Al^{3+} and Fe^{3+} aquo-ions respectively. The fraction

Table 1

Ultrafiltration of transferrin Fe^{III} Al^{III} mixtures

Total $[Al^{III}]$ (μM)	Total free $[Fe^{III}]$ (μM)[a]	Bound $[Fe^{3+}]$ (μM)[a]
250	2.4	17.6
1250	6.4	13.6
2500	9.4	10.6
5000	10.4	9.6

[a]Mean of two determinations
$[Ci]°$, 12.5 mM; $[Fe^{3+}]°$, 20 μM; $[HCO_3^-]°$, 6.7 mM: [transferrin]°, 15.6 μM

of the total binding sites occupied by ferric ions, θ_{Fe}, and the total unbound ferric ion concentration may be measured directly. However, it is necessary to calculate the free $[Al^{3+}]$ and $[Fe^{3+}]$ using the appropriate stability constants [6] for the formation of the citrate complexes of the latter ions at the known pH and total citrate concentration. The free $[Fe^{3+}]$ and $[Al^{3+}]$ in the ultrafiltrate are constrained by the following conservation equations where $[Fe^{III}]°$, $[Al^{III}]°$ and $[Ci]°$ represent total ferric, aluminium and citrate concentrations, respectively.

$$[Al^{III}]° = [Al^{3+}] + [AlOH^{2+}] + [Al(OH)_2^+] + [Al(OH)_3] + [Al(OH)_4^-] + [AlCi] + [AlHCi^+] + [AlH_{-1}Ci^-] \quad (2)$$

$$[Fe^{III}]° = [Fe^{3+}] + [FeOH^{2+}] + [Fe(OH)_2^+] + [Fe(OH)_4^-] + [FeH_{-1}Ci^-] + [FeH_{-2}Ci_2^{5-}] \quad (3)$$

$$[Ci]° = [AlCi] + [AlHCi^+] + [AlH_{-1}Ci^-] + 2[FeH_{-2}Ci_2^{5-}] + [Ci^{3-}] + [HCi^{2-}] \quad (4)$$

Multinuclear species are not included in view of the low aquo-ion concentrations expected in the presence of excess citrate.

The literature values at pH 7.4 for the relevant formation and dissociation constants for hydrolysis of Al^{3+} [10], aluminium citrate [11], Fe^{3+} [10], ferric citrate [6] and for citric acid dissociation [11], enable the $[Ci]°$ to be expressed as a polynomial of degree 4 in terms of $[Ci^{3-}]$ and solved by re-iteration with a Cyber 173 computer. This then permits direct calculation of the free

Table 2

The calculated free aquo-ion concentrations for Fe^{3+} and Al^{3+}, and the corresponding values for the effective aluminium-transferrin association constant, K'_{Al}, taken from the four pairs of data in fig. 3 where interference with Fe^{3+} binding was observed

$1/\theta_{Fe}$	$[Fe^{3+}] \times 10^{21}$ (M)	$[Al^{3+}] \times 10^{14}$ (M)	$K' \times 10^{-15}$ (M^{-1})
1.773	1.618	1.322	2.76
2.294	4.566	7.043	2.50
2.943	7.569	15.33	2.87
3.250	10.52	37.24	1.91

aquo-ion concentrations, and the corresponding K'_{Al} values can be derived using Eqn (1) (table 2), assuming $K'_{Fe} = 3 \times 10^{22}$ M^{-1} at pH 7.4 with excess bicarbonate [7], yielding a value (± S.D.) for K'_{Al} of 2.5 (± 0.4)$\times 10^{15}$ M^{-1}. It is reasonable to believe that this value should apply to human serum. Since transferrin is usually only 30% saturated with iron [12] it can be regarded as a potent ligand for aluminium under physiological conditions, utilizing the unoccupied sites. This is consistent with the fact that the serum transferrin concentration in dialysis patients is about 30 μM [13] and the maximum serum aluminium concentration observed is about 20 μM [14]. While the much lower value of K'_{Al} compared to K'_{Fe} makes it unlikely that aluminium could affect iron transport in serum, it is possible that transferrin could carry aluminium to sites where interference with iron metabolism might occur.

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