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An *in vitro* study on the binding of Al(III) to human serum transferrin with the isoelectric focusing technique

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Transferrin saturated with Al^{3+} subjected to isoelectric focusing (IEF) in a pH gradient can be separated into four fractions, representing the apotransferrin, transferrin with aluminum at the metal binding site in the C- or N-terminal lobe, or both. The electrophoretic mobilities of these four fractions are identical to those of the iron–transferrin counterparts. Simultaneous binding of aluminum and iron to transferrin can also be demonstrated. The decreased saturation after IEF indicates that the affinity of transferrin for aluminum is low compared with its affinity for iron. This effect is particularly evident when bicarbonate is used as the synergistic anion in the loading procedure. In contrast, loading of transferrin with aluminum in the presence of oxalate produces a di-aluminum–transferrin complex that is stable during IEF.

Keywords: aluminum, isoelectric focusing, PhastSystem, transferrin

Introduction

With the isoelectric focusing (IEF) technique we have demonstrated that transferrin isolated from human serum is very heterogeneous (de Jong *et al.* 1988, de Jong & Eijk 1990, van Eijk & van Noort 1992). This structural diversity is due to:

(1) Genetically determined variation of the polypeptide chain, e.g. TfC_1, TfC_2, TfB and TfD.
(2) Variation in the iron content, Tf, $Fe\text{–}_NTf$, $Tf\text{–}Fe_C$ and $Tf_2\text{–}Fe$.
(3) Variation in the *N*-linked glycan chains (asialo→ octasialo-Tf).

The latter has been termed microheterogeneity and can reliably be quantified by means of crossed immuno-IEF (de Jong & van Eijk 1988, de Jong 1993).

The percentage difference in isoforms can be determined and this can be applied in a diagnostic setting (van Eijk *et al.* 1987, de Jong *et al.* 1990).

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A great number of alternative metal ions can also bind to transferrin, albeit with lower affinity: Cr^{3+}, Cu^{2+}, Mn^{2+}, Co^{3+}, Zn^{2+}, Ga^{3+}, Ni^{2+} and Al^{3+}. Because transferrin under normal physiological conditions in serum is only partially (30%) saturated with iron, enough binding places remain available for transport of other metal ions (Welch 1992, de Jong 1993), despite their lower affinities.

Although aluminum is the most abundant metal in the Earth's crust, this preponderance in nature is not matched by a physiological function, presumably because aluminum is not a transition metal. Accordingly, in subjects with normal renal function, both total body content and serum concentrations are very low (0.1–0.3 μM). The use of aluminum hydroxide as a phosphate binder in the gastrointestinal tract in patients with renal failure and the fact that dialysis fluids are inevitably contaminated with aluminum to varying degrees depending on the water purification technology applied locally causes this population to be particularly prone to the development of the so-called aluminum-related diseases. Increasing awareness of this problem and technical developments have greatly decreased the incidence of caricatural aluminum overload over the last decade and the issue has now switched to more subtle diseases at the bone level, interferences with parathyroid function and resistance to erythropoietin

therapy (Consensus Conference 1993). In dialysis patients a serum aluminum level of $100\,\mu g\,l^{-1}$ is indicative of the presence of aluminum-related diseases like microcytic anemia, vitamin D resistant osteomalacia and dialysis encephalopathy. Aluminum has been implicated in the pathogenesis of some neurological diseases in subjects with normal renal function, but its definite role in such diseases like morbus Alzheimer, amyotrophic lateral schlerosis and Parkinson dementia remains to be determined.

Like iron, in the circulation, aluminum is transported by transferrin, while albumin and citrate appear to play a minor role (van Ginkel *et al.* 1990, Harris & Sheldon 1990). Competition between iron and aluminum for transferrin binding is suggested in view of the negative correlation between serum iron and serum aluminum in the dialysis population (Vanuytsel *et al.* 1992, van Landeghem *et al.* 1994). This, together with the assumption that aluminum enters the cell via transferrin receptor mediated endocytosis, explains the recent revival of interest in the aluminum binding properties of transferrin. Here, this has been studied by IEF of aluminum-loaded transferrin, as IEF has proven a successful technique with respect to the quantitative analysis and isolation of transferrin subspecies defined by differences in iron content (de Jong 1993). As the difference in the isoelectric point of these subspecies is determined by the exchange of hydrogen ions and binding of an anion in the metal binding site, it follows that IEF should be similarly instrumental in the separation of transferrin species differing in aluminum loading. It may distinguish whether aluminum is bound to either the metal binding site in the N- or C-lobe of transferrin ($_N$Tf or Tf_C), or to both sites, and this assumption has been tested, with a view to developing a method to detect transferrin-bound aluminum in serum by combining this method with mass spectrometry.

Methods and materials

Chemicals

All chemicals used were of p.a. grade. Aluminum was from Baker (Philipsburg, USA), human transferrin was from Behringwerke (Marburg, Germany) and all other chemicals were from Merck (Germany). Purified bibi-antennair transferrin was prepared in our laboratory (de Jong 1993).

Loading of transferrin

Transferrin (2 mg) was dissolved in 800 μl 50 mM Tris–HCl, pH 8.0. Subsequently, 50 μl of each of the following four solutions, 5 mM KCl, 5 mM NTA, 5 mM citrate and 5 mM oxalate, were added. After adjusting the pH to 8.0 we added Al^{3+} in the form of $AlCl_3$ in a 10-fold molar excess (7 μl). Loading in the presence of HCO_3^- was performed as previously described in detail for ^{56}Fe and ^{59}Fe (van Eijk & van Noort 1992, van Eijk *et al.* 1994).

In a number of publications absorption at 240 nm has been taken to reflect the formation of the Al–Tf complex and as such this was used to monitor the binding of aluminum to transferrin (Trapp 1983, Cochran *et al.* 1984, Harris & Sheldon 1990, McGregor *et al.* 1990, Kubal *et al.* 1992, Aramini *et al.* 1993). Based on this assumption, maximal absorption at 240 nm was assumed to be correlated with full saturation.

Techniques

IEF was carried out in the PhastSystem as previously described in detail (van Eijk & van Noort 1992, van Eijk *et al.* 1994). For relative quantifications of the transferrin bands we used a laser densitometer (van Eijk & van Noort 1992, van Eijk *et al.* 1994). The Al^{3+}/Tf ratio (see Table 2) is based on the calculated amounts of the components.

Results and discussion

As expected, after binding of Al^{3+} to human transferrin with oxalate an electrophoretic distribution picture similar to that found after saturation of transferrin with iron is obtained (Figure 1), indicative of the qualitatively identical bonding to the metal binding site. The same argument cannot be held with respect to the quantitative aspect, i.e. the strength of the interaction. Despite having followed the methods described for the saturation of transferrin with Al^{3+} (van Ginkel *et al.* 1990, Harris & Sheldon 1990, Consensus Conference 1993, Trapp 1983), in the IEF technique the reported full saturation with these procedures was not confirmed, as is shown in Table 1 and Figure 2. After IEF in ampholine gels Tf_2–Al is not found in the samples prepared following any one of the four loading protocols in which bicarbonate was added as the synergistic anion.

Following observations on the use of oxalate as the synergistic anion in Tf–Al binding (Aramni *et al.* 1993, Seidel *et al.* 1994), IEF of transferrin saturated with aluminum in the presence of oxalate was performed. In the presence of

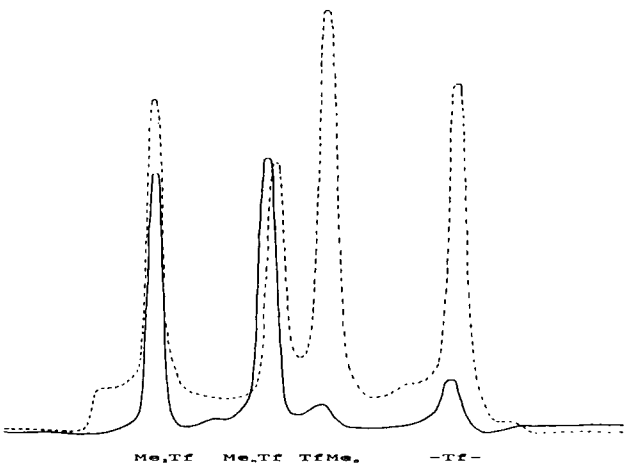

Figure 1. Laserscan comparing the IEF points of the isoforms of Fe^{3+}–Tf (– – –) and Al^{3+}–Tf (——) on an IEF gel. Anion, oxalate; Me, metal.

Table 1. Distribution of aluminum over the transferrin binding sites, following IEF of 100% saturated Al–Tf on ampholine gels (loading protocols are indicated by references in the left column)

Author	Al^{3+}/Tf molar ratio	apo-Tf (%)	Tf_C (%)	$_NTf$ (%)	Tf_2 (%)	Saturation after IEF (%)
Trapp 1983	2	80	14	6	—	10
Cochran *et al.* 1984	4	77	21	2	—	1
McGregor *et al.* 1990	2	96	4	—		2
Harris & Sheldon 1990	2	97	1	2	—	1.5

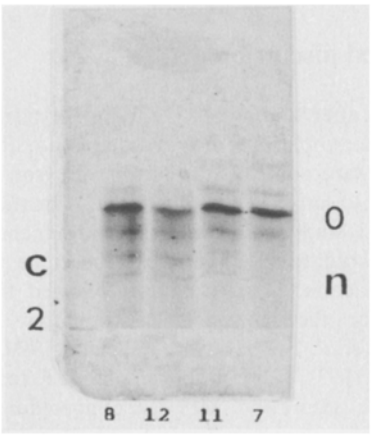

Figure 2. O, apo-transferrin; c, Tf–Al_C; n, Al–$_N$Tf; 2, Tf_2–Al. IEF gel of Al–Tf which is loaded according to four methods from the literature employing bicarbonate as the synergistic anion. High amounts of apoTf and less Tf–Al_C, Al–$_N$Tf and Tf_2–Al are found after IEF of Tf_2–Al solution.

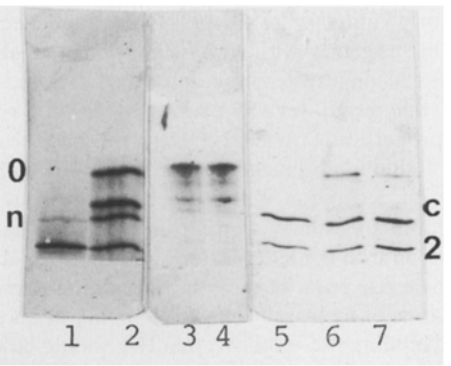

Figure 3. O, c, n and 2, see legend to Figure 2. Lane 1, Fe–Tf, loaded with oxalate as anion, Fe–$_N$Tf and Tf_2–Fe are abundant. Lane 2, Fe–Tf, loaded with bicarbonate as anion, all the isoforms are visible. Lane 3, Al–Tf, loaded with bicarbonate at pH 8.0. Apo-Tf, Tf–Al_C and Al–$_N$Tf are visible. Lane 4, Al–Tf, loaded with bicarbonate at pH 7.4. Lane 5, Al–Tf, loaded with oxalate as anion, saturation has improved, only Al–$_N$Tf and Tf_2–Al are detected. Lane 6, Al–Tf, loaded with oxalate, reduced incubation temperature in comparison with sample 5. Lane 7, Al–Tf, same as sample 6, incubation time doubled. The apo-Tf band diminishes in favor of the Al_2–Tf band.

bicarbonate and an excess of Al^{3+} only apo-Tf and Tf–Al_C can be detected, while using oxalate as the synergistic anion the saturation after IEF is increased to 70% and Al–$_N$Tf and Tf_2–Al are also present. Results are summarized in Table 2 and visualized in lanes 3–7 of Figure 3.

After refocusing of bicarbonate–Tf–Al_C extracted from gels, mainly apotransferrin is recovered while, on the contrary, refocusing of oxalate–Tf_2–Al results in the approximate loss of only 3% of the aluminum. The fact that this complex is almost completely regained as the Tf_2–Al complex casts doubt on the acclaimed efficacy of the loading

Table 2. Distribution of the Al–Tf forms under different incubation conditions

Lane in Figure 3[a]	O (%)	Tf_C (%)	$_NTf$ (%)	Tf_2 (%)	Saturation (%)	Incubation (h)	Temperature (°C)	Anion	Al^{3+}/Tf molar ratio during incubation
3	68	19	13	—	16	24	20	bicarbonate	12
4	85	15	—	—	7.5	24	20	bicarbonate	12
5	—	—	69	31	66	24	37	oxalate	10
6	24	—	54	22	49	24	20	oxalate	10
7	6	—	50	44	69	48	20	oxalate	10

[a] Lanes 3–7, transferrin loaded with Al^{3+}.

Table 3. Completion of Al–Tf saturation with Fe^{3+} (appearance of a dimetallic Al + Fe–Tf fraction at the cost of disappearance of the mono Al–Tf fraction)

Lane in Figure 4	O (%)	Tf_C (%)	$_NTf$ (%)	Tf_2 (%)	Saturation (%)	Added Fe^{3+} (nmol)
1			40	60	80	
2			22	78	89	1.0
3			10	90	95	2.5
4				100	100	5.0

procedures described in the literature, since a fair amount (< 10%) of Al–$_N$Tf was detected in the samples taken directly from the loading buffer.

As an additional experiment, increasing amounts of iron were added to a solution containing approximately 80% saturated Tf–Al. In the electrophoretic patterns we noted disappearance of Al–$_N$Tf and appearance of an Al–Tf–Fe band. The latter is inferred from stoichiometric considerations; only just enough iron was added to fill the remaining open sites. Results are shown in Table 3 and Figure 4.

Taken together, these results indicate that with bicarbonate as the synergistic anion, aluminum is coupled to transferrin with insufficient affinity to be able to withstand chelating properties of ampholines during migration through the gel, possibly due to destabilization of the bond as a result of the local pH in the gel. In contrast, under present focusing conditions oxalate–Tf_2–Al is a virtually stable complex. Although a 1000-fold excess of bicarbonate over oxalate exists in plasma, the much higher affinity of aluminum for transferrin in the presence of oxalate suggests that this may be the prevailing complex *in vivo*.

A more detailed study of aluminum metabolism in plasma applying the electrophoretic techniques used in this study thus appears to be feasible. However, since the amount of Fe–Tf complexes will far outweigh their aluminum counterparts, and that this work has demonstrated the possibility of existence of mixed metal–transferrin complexes, electrophoretic patterns are identical with those of the pure metals. This would necessitate a distinction of the nature and amounts of different metals found in each of the electrophoretic variants found in serum. In principle this is possible as the mono-metal– and di-metal–transferrin variants can be isolated from the sample by preparative IEF (van Eijk *et al.* 1980). As a preliminary experiment we have attempted to detect an aluminum signal by atomic absorption spectrometry from the three metal-containing subfractions isolated from the PhastGel after IEF of oxalate-saturated transferrin, but were unable to detect this signal. The latter is most likely due to the very small amount of metal present in these isolated transferrin fractions (only 2 μl was applied, limiting the amount of metal in samples to the lower picomole range).

Whether similar occupation of binding sites occurs *in vivo*, particularly in view of the 1000-fold excess of HCO_3^- over oxalate in serum *in vivo*, thus remains to be established and awaits a more sensitive method for the measurement of aluminum in PhastGel or similar media.

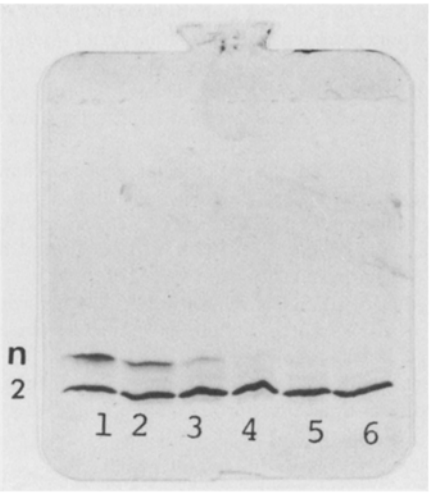

Figure 4. IEF of a transferrin (2.75 mg ml^{-1}) solution containing Al–$_N$Tf (40%) and Tf_2Al (60%). An increasing amount of Fe^{3+} is added. The Al–$_N$Tf band decreases in favor of the Al–$_N$Tf–Fe_C band. Anion, oxalate. Lane 1, no Fe^{3+} added. Lane 2, 1 nM Fe^{3+} added. Lane 3, 2.5 nM Fe^{3+} added. Lane 4, 5 nM Fe^{3+} added. Lane 5, 20 nM Fe^{3+} added. Lane 6, 100 nM Fe^{3+} added.

Further analysis on patient material is pending, awaiting this technical development, but given such a method in the future serum aluminum turnover characteristics and metabolism can be studied in more detail, as in principle occupation of iron-binding sites by aluminum in the presence of non-saturating levels of iron has now been estalished.

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