Trace Elements

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Binding of Chromium(III) to Transferrin Could Be Involved in Detoxification of Dietary Chromium(III) Rather than Transport of an Essential Trace Element

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Abstract: *Cr^{III} binding to transferrin (Tf; the main Fe^{III} transport protein) has been postulated to mediate cellular uptake of Cr^{III} to facilitate a purported essential role for this element. Experiments using HepG2 (human hepatoma) cells, which were chosen because of high levels of the transferrin receptor, showed that Cr^{III} binding to vacant Fe^{III}-binding sites of human Tf effectively blocks cellular Cr^{III} uptake. Through bio-layer interferometry studies of the Tf cycle, it was found that both exclusion and efflux of $Cr_2^{III}Tf$ from cells was caused by 1) relatively low Cr_2Tf affinity to cell-surface Tf receptors compared to Fe_2Tf, and 2) disruption of metal release under endosomal conditions and post-endosomal Tf dissociation from the receptor. These data support mounting evidence that Cr^{III} is not essential and that Tf binding is likely to be a natural protective mechanism against the toxicity and potential genotoxicity of dietary Cr through blocking Cr^{III} cellular accumulation.*

Since Cr^{III} was first proposed to be essential for glucose metabolism in the 1950s,[1] its popularity as a dietary supplement both by itself and in multivitamins has grown dramatically. However, there is a growing consensus that Cr^{III} lacks specific biological functions in mammals.[2] In addition, new evidence indicates that excessive consumption of Cr^{III} may pose health risks owing to the generation of carcinogenic Cr^{VI} and Cr^{V} species in vivo.[3a–d] The idea that Cr is an essential trace element led to the hypothesis that Cr^{III} transport from blood to cells occurs through binding to Fe^{III}-binding sites of transferrin (Tf), followed by receptor-mediated endocytosis.[4] More generally, Tf has been postulated to mediate cellular uptake of metal ions other than Fe^{III} [4b,5] for a number of reasons: 1) efficient binding of many transition-metal ions, particularly trivalent ones, to the Fe^{III} binding sites of apo-Tf,[5] 2) partial Fe^{III} saturation of Tf in vivo (large variations amongst individuals; the average is ca. 30%),[6] which leaves vacant sites for other metals to bind,[4b,5] and 3) a highly efficient cellular uptake mechanism for Fe_2Tf (the Tf cycle)[7] that is presumed to accept other metal Tf complexes.[4b] However, recent studies[8,9] have demonstrated that loading of apo-Tf with metal ions other than Fe^{III} results in disruption of binding to the Tf receptor 1 (TfR1) at the cell surface, which is a crucial step in the Tf cycle.[7] To our knowledge, no direct studies of $Cr^{III}Tf$/TfR1 binding have been performed to date.[4b,8] Herein, we report on the role of Tf in the cellular uptake of Cr^{III} by using our recently developed bio-layer interferometry (BLI)[9] method for studying the Tf cycle in a cell-free environment, in combination with Cr^{III} uptake studies in cultured human liver cancer cells (HepG2 cells, which are rich in TfR1).[10] Such binding strongly inhibits rather than facilitates Cr^{III} uptake. These data represent a new and important contribution to a series of recent results that are dismantling the postulated mechanisms associated with the putative essential biological role of Cr.[2,3]

Cell culture media for Cr uptake experiments (Table 1 and the Supporting Information) were prepared by adding human Tf (0%, 30%, or 100% Fe^{III}-saturated) and/or Cr^{III} to base medium [Dulbecco's modified minimal essential medium (DMEM) with 0.1% mass bovine serum albumin (BSA)]. The absence of added serum in the medium ensured the absence of adventitious Tf, while the presence of a minimal amount of background protein (BSA) was required for cell survival during the treatments.

All experimental numbers refer to Table 1 (30 μM [Tf]; typical for human blood serum).[6] The control had human serum albumin (HSA, 30 μM) instead of Tf (entry 7). Freshly prepared aqueous $CrCl_3 \cdot 6H_2O$ stock solution (60 μM; predominantly *trans*-$[Cr^{III}Cl_2(OH_2)_4]^+$)[11] was added to the medium to form Cr_2Tf stoichiometrically.[12] As an additional control, isolated Cr_2Tf (characterized by UV/Vis spectroscopy,[12] Figure S1 in the Supporting Information) was added to the base medium (entry 8). The resulting media were equilibrated (24 h, 310 K, 5% CO_2) prior to cell treatment to ensure full aquation of *trans*-$[Cr^{III}Cl_2(OH_2)_4]^+$ and its binding to medium components, including added proteins.[13] Cellular Cr content was determined by graphite furnace atomic absorption spectroscopy (GFAAS) of cell lysates after 24 h incubation with pre-equilibrated media.

Pre-equilibration of 60 μM Cr^{III} in base medium resulted in a approximately 1% uptake of total Cr by HepG2 cells after a 24 h incubation (entry 3), but this technique does not distinguish between intracellular and membrane-bound Cr, so the Cr content may reflect Cr^{III} absorption with medium components on the cell surface.[13] Addition of apo-Tf or partially Fe^{III}-saturated Tf (ca. 30%) to this medium significantly reduced the cellular Cr content (entries 4 and 5). Surprisingly, the largest decrease occurred when isolated Cr_2Tf (30 μM) was added to the medium instead of 30 μM Tf/60 μM Cr^{III} (entry 8). Replacement of Tf with HSA (a non-specific Cr^{III} binder)[13,14] resulted in a slight decrease in Cr uptake compared to that from the base medium (entries 3 and

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Table 1: Conditions and results of cell treatments and BLI measurements.

Entry	Additions to base medium[a]	Cr in cells[b] [nmol mg^{-1}]	Cr in cells[c] [mol %]	HMM Cr[d] [mol %]	Fe in cells[e] [nmol mg^{-1}]	BLI of cell culture medium[f] K_{D1} [nM]	K_{D2} [nM]	Source
1	30 μM Fe_2Tf, no Cr^{III}	<0.1[h]	–	–	42 ± 4***	1.5 ± 0.1	7.8 ± 0.5	[f]
						0.61	6.3	Ref. [16a][l]
						0.72 ± 0.6	4.1 ± 1.4	Ref. [16b][l]
2	30 μM apo-Tf, no Cr^{III}	<0.1[h]	–	–	<2.5[i]	5.1 ± 0.6	50 ± 5	[f]
3	60 μM Cr^{III}	1.9 ± 0.4	1.0 ± 0.2	9.9 ± 2.5	<2.5[i]	$>10^4$[j]	–	[f]
4	30 μM apo-Tf + 60 μM Cr^{III}	0.10 ± 0.03	0.05 ± 0.02	75 ± 8***	<2.5[i]	4.8 ± 0.3	23 ± 2	[f]
5	30 μM $Fe_{0.6}Tf$[g] + 60 μM Cr^{III}	0.15 ± 0.04	0.07 ± 0.02	59 ± 2***	<2.5[i]	5.2 ± 0.5	18 ± 2	[f]
6	30 μM Fe_2Tf + 60 μM Cr^{III}	13 ± 2***	6.4 ± 1.1***	8.2 ± 1.6	45 ± 3***	2.3 ± 0.2	10 ± 1	[f]
7	30 μM HSA + 60 μM Cr^{III}	1.2 ± 0.2	0.6 ± 0.1	11.5 ± 2.4	<2.5[i]	300 ± 20[k]	–	[f]
8	30 μM Cr_2Tf	<0.1[h]	<0.05[h]	73 ± 6***	<2.5[i]	3.9 ± 0.3	18 ± 2	[f]

[a] Base medium: serum-free DMEM with 1.0 mg mL^{-1} BSA; the base medium contained 0.10 ± 0.05 μM Cr and 6 ± 2 μM Fe, respectively (by GFAAS). For entries 3–7, Cr^{III} = *trans*-$[Cr^{III}Cl_2(OH_2)_4]^+$ (freshly prepared aqueous stock solution). All Cr-supplemented media were equilibrated (24 h, 310 K, 5% CO_2) before incubation with HepG2 cells for 24 h. [b] Cr levels in cell lysates (nmol Cr mg^{-1} protein). Cr and Fe levels in cell lysates are given as the mean ± SD ($n=6$, 2 independent experiments with 3 replicates each). *** Highly significant ($P<0.001$; one-way ANOVA test) increases in metal binding compared with entry 3. [c] The Cr level in cell lysates (mol % of total Cr added to the medium). [d] Part of total Cr (mol %) bound to the >6 kDa fraction of cell culture medium (HMM = high molecular mass) determined by gel filtration chromatography and GFAAS. [e] Fe content in cell lysates (nmol Fe mg^{-1} protein). [f] This work: aliquots of cell culture media collected after cell treatments and diluted 30-fold with the background buffer (20 mM HEPES, 25 mM $NaHCO_3$, 140 mM NaCl, 2.0 mg mL^{-1} BSA, pH 7.40). Binding affinities of resulting solutions to immobilized human TfR1 were measured using BLI.[9] K_D values are given as the mean ± SD of 3 measurements with different BLI probes (kinetic analyses and K_D calculations, Table S1). [g] Partial Fe^{III} saturation of Tf (≈30%), corresponds to typical blood conditions in vivo.[6] [h] The Cr level was below the GFAAS detection limit. [i] The Fe level in the cell lysate was not significantly higher than in the blank lysis solution (0.10 M NaOH). [j] BLI probe binding was not significantly different from that of the background buffer. [k] Low-affinity HSA binding to TfR1-coated BLI probes (Table S1, Figure S2). [l] Fe_2Tf binding to TfR1 in aqueous buffers (rather than in cell culture medium) at pH 7.4 was measured by surface plasmon resonance methods.

7). By contrast, a highly significant increase in cellular Cr content was achieved upon the addition of Fe^{III}-saturated Tf (Fe_2Tf) to Cr^{III}-containing cell culture medium (entry 6).

Strong binding of Cr^{III} to Fe^{III}-free or partially Fe^{III}-saturated Tf, but not to fully Fe^{III}-saturated Tf, was confirmed by gel filtration of aliquots of cell culture media after incubation with cells (the proportions of protein-bound Cr^{III} are shown in Table 1, column 5). Cr^{III}-protein binding in the medium with added Fe_2Tf was no higher than in the medium with no added Tf (entries 3 and 6), which suggests only transient Cr^{III} binding to Fe_2Tf. Intracellular Fe significantly increased in the presence of added Fe_2Tf (30 μM, entries 1 and 6, which was consistent with active TfR1-mediated Fe^{III} uptake by HepG2 cells.[7–10] In summary, these experiments demonstrate that the binding of Cr^{III} to the vacant Fe^{III} binding sites of human Tf blocks rather than promotes Cr^{III} accumulation by human liver cells. By contrast, adventitious binding of a small proportion of Cr^{III} to the surface of Fe_2Tf led to a prominent increase in cellular Cr and Fe contents (entry 6). The latter finding is consistent with the data on protein binding and cellular uptake of medicinal Ru^{II}, Pt^{II}, and V^{IV} complexes in the presence of Fe_2Tf.[15]

Aliquots of cell culture media collected after incubation with HepG2 cells were also used for BLI analysis of Tf/TfR1 binding and dissociation (after dilution to [Tf] = 1.0 μM, Table 1 and Table S1 in the Supporting Information).[9] Typical BLI data (Figure 1 a) provided the first complete cell-free model of the Tf cycle,[7] including: binding of Fe^{III}Tf from blood plasma to TfR1 at the cell surface (step A); endosomal cellular Fe_2Tf/Tf1 uptake (step B); endosomal acidification (step C) to release Fe^{III} and form the apo-Tf/TfR1 complex (stable at pH 5.60); and the return of the complex to the cell surface and its rapid dissociation (pH 7.40, step D).[9] Detailed kinetic analysis of BLI models of the Tf cycle will be published elsewhere.

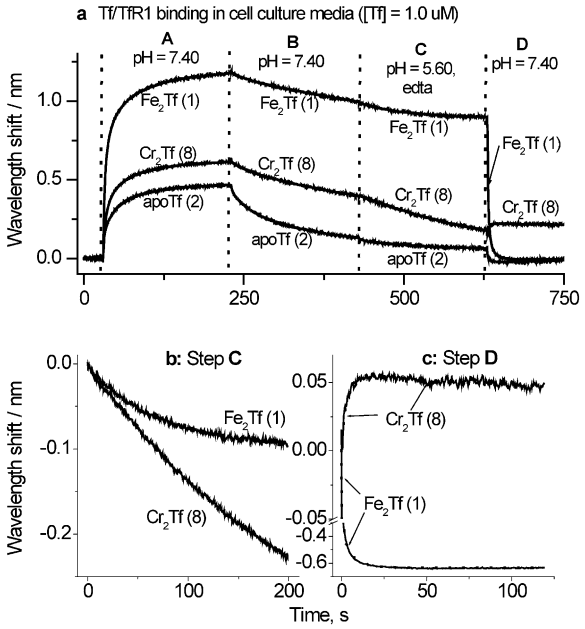

Figure 1. Typical BLI results (295 K, a: full cycle; b,c: for comparison separate steps were aligned to start from zero)[9] for dilute cell culture media after HepG2 cell incubations. Corresponding entry numbers for table 1 are given in parentheses. Media aliquots were diluted 30-fold with background buffer (20 mM HEPES, 25 mM $NaHCO_3$, 140 mM NaCl, 2.0 mg mL^{-1} BSA, pH 7.40) immediately prior to binding to immobilized TfR1 on a BLI probe (step A). The same buffer was used for dissociation steps B and D. The endosomal buffer (step C)[9,18] had 100 mM MES, 300 mM KCl, 4.0 mM Na_2edta, and 2.0 mg mL^{-1} BSA; pH 5.60. Parts (b) and (c) show experimental (solid) and fitted (dotted) kinetic curves. BLI results for all the experiments (Table 1) and details of kinetic analyses are given in Table S1.

Binding of Cr_2Tf (Figure 1a, steps A and B, and Table 1, entry 8) to a TfR1-coated BLI probe at pH 7.40 was stronger than that of apo-Tf (entry 2), but weaker than that of Fe_2Tf (entry 1). Kinetic analysis of steps A and B using a 2:2 binding model (two Tf molecules bind sequentially to TfR1 at the cell surface, Table S1)[16] gave the dissociation constants (K_{D1} and K_{D2}, Table 1). K_D values for Fe_2Tf/TfR1 binding at pH 7.40 (Table 1, entry 1) were close to those obtained[16] using a related but not equivalent surface plasmon resonance method.[9] A slight decrease in Fe_2Tf/TfR1 binding affinity compared with published data[16] (entry 1) was probably due to components of the cell culture media (e.g., phosphate and amino acids) that compete with Tf for Fe^{III} binding.[17]

The largest change in the Fe_2Tf/TfR1 and Cr_2Tf/TfR1 binding cycles was substantial inhibition of post-endosomal Tf dissociation (step D; Figure 1a) for Cr (Figure 1c and Figure S2). This arises from a difference in Cr_2Tf and Fe_2Tf dissociation kinetics (step C, Figure 1) under acidic endosomal-mimetic conditions that lead to Fe^{III} release from Tf/TfR1.[9,18] Cr^{III} release from kinetically inert Cr_2Tf will be slow under endosomal-mimetic conditions;[13] the first-order dissociation in step C (Figure 1b and Figure S2) is assigned to Cr_2Tf dissociation from TfR1. By contrast, the biphasic dissociation kinetics for Fe_2Tf (step C; Figure 1b and Figure S2) is assigned to conformational changes resulting from Fe^{III} release from the Tf/TfR1 complex, followed by slow dissociation of apo-Tf from TfR1 at pH 5.60.[7,18] The BLI results were similar for all the cell culture media that contained Cr^{III} bound to the Fe^{III} binding sites of Tf (entries 4, 5 and 8), while those for Cr^{III} bound to the Fe_2Tf surface (entry 6) were not significantly different from those from Cr^{III}-free Fe_2Tf (entry 1, see Table S1, Figure S2).

The data indicate that the main reason for the low cellular accumulation of Cr_2Tf is disruption of endosomal and post-endosomal steps of the Tf cycle (steps C and D; Figure 1 and Table 1),[9,18] but decreased Cr_2Tf/TfR1 binding affinity compared with Fe_2Tf (steps A and B; Figure 1a)[9,16] also plays an important role. The post-endosomal dissociation (step D) of Cr_2Tf was slower than the pre-endosomal process (step B), despite the same pH and buffer conditions (Figure 1a). This difference is likely to be due to cross-linking of Tf to TfR1 by Cr^{III} released under slightly acidic conditions during step C.[9,13]

The results show that Cr^{III}, like the non-essential Al^{III},[19] does not undergo the postulated[4] active Tf transport into human cells, despite efficient binding to the Tf Fe^{III} binding sites.[5] On the contrary, Cr^{III} binding to the vacant binding sites of partially Fe^{III}-saturated Tf under physiological conditions may serve as a Cr^{III} detoxification mechanism[4b,c] to reduce toxicity[2b3] from dietary and supplemental intake of Cr^{III}. On the other hand, adventitious uptake of Cr^{III} bound to the side chains of Fe_2Tf is not excluded,[15] since such binding does not disrupt cellular uptake of Fe_2Tf by receptor-mediated endocytosis (entry 6).

The question arose as to why Cr^{III} binding to typical distributions of apo-Tf, FeTf and Fe_2Tf in blood serum[6] (entry 5) did not result in large increases in cellular Cr from any Cr binding to the protein surface of the Fe_2Tf component. From entry 6, this was expected to result in 1–3 nmol mg^{-1} of cellular Cr from typical levels of Fe_2Tf within blood plasma, whereas the actual cellular Cr content was an order of magnitude lower. This effect is explained by Cr_2Tf and CrFeTf binding to TfR1 not only interfering with Cr uptake but also the entire Tf cycle, and this was confirmed by substantial suppression of Fe uptake from that expected for the amount of Fe_2Tf present. Cr-induced interference with the Fe uptake cycle also explains anemia induced by high consumption of chromium picolinate.[20] These results also suggest that insulin-mediated cellular uptake of Cr^{III}, which was previously taken as a sign of Cr being essential,[4] is more likely to be linked to the role of insulin as a promotor of Fe_2Tf uptake through the Tf cycle.[21] Moreover, these results provide a physiological rationale for the partial saturation of circulating Tf. Instead of the postulated role as a mechanism of cellular uptake of other trace elements, this could be a mechanism for binding and preventing cellular accumulation of metal toxins.

Efficient uptake of Cr^{III} by rat tissues[4] after intravenous injection of ^{51}Cr-labelled Cr_2Tf can be explained by a variety of alternative mechanisms, including 1) dissociation of Cr_2Tf in the blood and binding of Cr^{III} to low-molecular-mass components of serum, followed by cellular uptake of the resulting complexes by passive diffusion,[4b,13,19] and 2) diffusion through junctions between the cells (paracellular uptake as described for Al^{III}).[19] Cr^{III} can also be oxidized in blood plasma under oxidative stress conditions to Cr^{VI}, followed by efficient cellular uptake through phosphate and sulfate channels[3,13] and transport into the nucleus through electrostatic binding to histone proteins.[22]

As well as low-affinity M_2Tf/TfR1 binding[8,9] ($M^{III} \neq Fe^{III}$), disruption of the endosomal and post-endosomal steps of the Tf cycle is a major reason for low cellular M accumulation.[7,18] In addition, unlike Fe^{III},[7] Cr^{III} endosomal reduction to M^{II} is not expected[3b,13] in order to enable its removal from the endosome through the M^{II} transporter channel (a suggested Fe^{II} transport mechanism).[7] This difference in redox properties is not modeled by BLI experiments but is a likely additional reason for poor Cr^{III} cellular uptake in the presence of Tf (Table 1). Finally, binding by HSA (entry 7), which is the other major serum protein that binds Cr^{III},[23] also inhibits Cr^{III} uptake by cells.

In summary, these results support the status of Cr^{III} as a nonessential metal ion for humans,[2] since it would most likely be transported from food in the GI tract to Tf in the blood. This is supported by other evidence: 1) the purported chromodulin may be an artifact,[24] 2) Cr^{III} supplements only have biological activities at levels much higher than those obtained from the diet,[2] and 3) mechanisms of activity appear to involve extracellular and intracellular oxidation to Cr^{V} and Cr^{VI}, followed by phosphatase inhibition.[3,13] The latter presents a potential health hazard (resulting from the oxidation of Cr^{III} to carcinogenic Cr^{VI} under biological conditions).[3] While no long-term human trials have been conducted over the 10–40 year timescale required to assess the risk of Cr^{III}-induced toxicity and cancers,[25] circulating Cr^{III} levels derived from surgical implants, which may be comparable to those obtained from Cr supplementation, result in a range of chronic toxicities.[25] Moreover, signifi-

cantly increased Cr levels have been found in the hair of breast cancer patients compared to matched controls.[26] Another small study found significantly higher levels of Cr and other metal toxins in breast cancers than in control tissues.[27] Finally, rat studies are often used to assess Cr^{III} (geno)toxicity, however, 1) the percentage of Cr^{III} absorbed from oral ingestion of Cr^{III} is many-fold higher in humans than in rats,[28] and 2) saturation of Cr^{III} accumulation in vital organs, such as the liver and kidney, is estimated to take more than 10 years with daily Cr supplementation,[3e] so rat studies almost certainly underestimate long-term human risks.

In summary, Cr supplementation over many years can result in bioaccumulation levels in vital organs that are orders of magnitude higher (low to mid μm levels) than baseline levels from dietary Cr.[3e] This bioaccumulation is typical for toxic heavy metals and is unlike the homeostatic control of essential trace elements in such organs. While the Cr_2Tf binding described herein is protective against dietary Cr bioaccumulation, it is clear from animal and human studies that this is partially overcome by long-term supplementation,[3e] with potential long-term toxicity.

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