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Phosphate inhibits *in vitro* Fe^{3+} loading into transferrin by forming a soluble Fe(III)–phosphate complex: A potential non-transferrin bound iron species

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ABSTRACT

In chronic kidney diseases, NTBI can occur even when total iron levels in serum are low and transferrin is not saturated. We postulated that elevated serum phosphate concentrations, present in CKD patients, might disrupt Fe^{3+} loading into apo-transferrin by forming Fe(III)–phosphate species. We report that phosphate competes with apo-transferrin for Fe^{3+} by forming a soluble Fe(III)–phosphate complex. Once formed, the Fe(III)–phosphate complex is not a substrate for donating Fe^{3+} to apo-transferrin. Phosphate (1–10 mM) does not chelate Fe(III) from diferric transferrin under the conditions examined. Complexed forms of Fe^{3+}, such as iron nitrilotriacetic acid (Fe^{3+}-NTA), and Fe(III)–citrate are not susceptible to this phosphate complexation reaction and efficiently deliver Fe^{3+} to apo-transferrin in the presence of phosphate. This reaction suggests that citrate might play an important role in protecting against Fe(III), phosphate interactions *in vivo*. In contrast to the reactions of Fe^{3+} and phosphate, the addition of Fe^{2+} to a solution of apo-transferrin and phosphate lead to rapid oxidation and deposition of Fe^{3+} into apo-transferrin. These *in vitro* data suggest that, in principle, elevated phosphate concentrations can influence the ability of apo-transferrin to bind iron, depending on the oxidation state of the iron.

1. Introduction

Iron is an essential element required to sustain life, but if not properly bound and directed to the correct locations in the body it can catalyze the formation of reactive oxygen species (ROS) [1]. To minimize ROS formation, nature has evolved mechanisms to transport, sequester, and escort iron to specific locations within the body. These mechanisms include membrane permeases, transport and storage proteins, hormones that regulate dietary iron absorption and redistribution, cytosolic iron chaperones and even a newly discovered mammalian siderophore [2–12]. In order to maintain healthy iron homeostasis, all of these mechanisms must be synchronized and function properly.

"Free iron", also known as non-transferrin bound iron (NTBI), represents a state where the normal iron processing mechanisms are unable to bind all of the iron that is present in biological fluids [13–18]. NTBI is a biomarker in iron overload diseases and of several other diseases such as diabetes and chronic kidney disease. Hemochromatosis and thalassemia patients have NTBI because iron-overload leads to serum iron concentrations that are higher than the iron binding capacity of serum iron binding proteins [19–21]. NTBI is also found in iron deficient or anemic individuals who receive iron supplements when the dose of administered iron is sufficient to exceed the binding capacity of serum iron proteins [20,22].

Recently, NTBI was identified in diabetes and CKD patients in whom iron was not in excess and serum transferrin was not saturated. These conditions existed even prior to iron supplementation [18–21,23]. This observation is puzzling because transferrin has a binding constant of ~10^{20} [24,25]. The occurrence of NTBI when transferrin is not saturated suggests that some NTBI exists as an iron species that is not biologically accessible to transferrin or indicates that an inhibitor is present that blocks iron binding to apo-transferrin.

Serum from patients with NTBI has been studied to characterize the molecular form of NTBI. Some fractions of NTBI are liberated by chelation using deferoxamine (DFO), but other fractions require oxalate, EDTA or nitrilotriacetic acid to mobilize the iron before DFO can complex the iron [14,26–28]. NMR studies and ultrafiltration studies have shown that some of the NTBI exists as low molecular mass complexes of citrate [29]. In contrast, a larger molecular mass fraction of NTBI exists that is presumably associated with proteins such as serum albumin [23]. The larger molecular mass NTBI may also be associated with phosphate species because larger molecular mass polymeric iron phosphate complexes have also been identified [30,31].

Bates et al. demonstrated that polymeric Fe(III) species are poor substrates for loading iron into apo-transferrin. [32,33]. In fact, many iron supplements are polymeric species and only transfer a small fraction

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of Fe(III) to transferrin, while cells lining the bloodstream absorb the rest of the iron supplement. However, this iron can be exported back to the bloodstream through ferroportin for efficient loading into transferrin [20,34,35]. Unfortunately, if patients suffer from an inflammatory disease, the iron hormone hepcidin causes ferroportin to be degraded [36]. The hepcidin-induced degradation of ferroportin prevents iron export from iron-rich cells and prevents iron loading into transferrin. Therefore, diseases with inflammatory conditions such as CKD and diabetes are ineffectively treated with iron supplements.

Phosphate is known to form polymeric iron species and phosphate concentrations are significantly elevated in the serum of CKD patients [37] (~3.6 mM) compared to healthy individuals (0.8–1.5 mM) [38]. These physiological conditions led us to postulated that the elevated phosphate concentrations might interfere with apo-transferrin iron binding by forming Fe(III)–phosphate complexes in the bloodstream of CKD patients [39–41]. The present study demonstrates that phosphate does in fact inhibit iron loading into apo-transferrin during *in vitro* iron loading reactions by forming Fe(III)–phosphate polymers, suggesting that Fe(III)–phosphate complexes may represent an important species of NTBI in serum.

2. Materials and methods

2.1. Materials

Human apo-transferrin was purchased from Sigma. Prior to iron loading, apo-transferrin (78 kDa) was prepared in a solution of 25 mM MOPS (3-(N-morpholino)propanesulfonic acid), pH 7.4. $Fe(NH_4)_2(SO_4)_2{\cdot}H_2O$ (Fe^{2+}), $FeCl_3$ (Fe^{3+}), Na_2HPO_4, and $NaHCO_3$, were all purchased from Fisher. Fe^{2+} and Fe^{3+} solutions were prepared by dissolving the appropriate solid into 0.001 M HCl. Nitrilotriacetic acid (NTA) was purchased from Sigma. The Fe–NTA complex was prepared as previously described [33,42] by adding $FeCl_3$ to an NTA solution so that a 1:4 ratio of Fe^{3+} to NTA was achieved. The solution was adjusted to pH 7.4 for the reactions.

2.2. UV/Vis spectrophotometry

An Agilent 8453 UV/Vis spectrophotometer was used to monitor the binding of iron to transferrin. Final concentrations of protein and solutions were: 5 mg/mL transferrin (~ 6.4×10^{-5} M), in 25 mM MOPS buffer pH 7.4, 10 mM CO_3^{2-}, phosphate concentrations as indicated in the figures, and either 0.18 mM Fe^{3+} or Fe^{2+}. The kinetic runs were setup to monitor the change in absorbance at 460 nm versus time. Transferrin was incubated with carbonate in the absence or presence of phosphate and allowed to stir to equilibrate. The kinetic run was initiated by adding the appropriate volume of Fe^{3+} or Fe^{2+}. Runs were collected in triplicate. Some reactions were initiated by adding $FeCl_3$ while other reactions were initiated by the addition of Fe^{3+}-NTA.

2.3. Difference spectra

The procedure of Gelb et al. was used to measure Fe^{3+} binding to the tyrosine residues of apo-transferrin by monitoring the deprotonation of the tyrosine residues [43]. Briefly, all solutions were prepared in 0.1 M KCl, 0.05 M $NaHCO_3$ adjusted to pH 7.4, the indicated concentrations of phosphate, and 2.0×10^{-5} M human apo-transferrin. Please note for comparison purposes that the carbonate concentration in the difference spectra reactions are 5 times higher than those in the UV/Vis spectrophotometry reactions and the apo-transferrin concentrations are ~3 times lower than the UV/Vis spectrophotometry reactions. The reaction solution (1.8 mL) was placed in a cuvette with a stir bar. 9.0 μL aliquots of a 1.0×10^{-3} M $FeCl_3$ solution were added to the apo-transferrin, for a total of 0.5 Fe/Tf per aliquot. The reaction was allowed to react for 1 minute. The sample spectrum was recorded and the next aliquot was added. The difference spectra were obtained by subtracting a control spectrum where water was added to the sample instead of iron.

Diferric transferrin iron release assays were performed by incubating diferric transferrin (42 μM) with 10 mM phosphate or 10 mM pyrophosphate. Iron release was detected by monitoring the decrease in absorbance at 460 nm as the reaction progressed with time.

3. Results and discussion

Transferrin specifically binds two Fe^{3+} ions, one in the N-terminal lobe (Fig. 1) and one in the C-terminal lobe [44–50]. Each lobe has slightly different structural properties that lead to slightly different affinity for Fe^{3+}. The differences between the lobes are proposed to be important in the iron binding and iron release mechanisms of transferrin [51]. Carbonate also binds in each of the lobes of transferrin [44,52] and acts as the synergistic anion to provide two coordinating ligands to anchor the iron into the binding cavity Scheme 1A. The proposed mechanism is that carbonate binds in the iron-binding pocket of each lobe to neutralize the positive charge of positively charged residues. Once bound, the carbonate provides the final two ligands for coordinating the Fe^{3+} ion [25].

One indicator of iron binding to transferrin is the characteristic absorbance peak at 460 nm that forms when iron is bound to transferrin. Fig. 2 shows the absorbance spectrum of apo-transferrin treated with Fe^{3+} and carbonate to form diferric transferrin. The control reactions show the spectrum of apo-transferrin, apo-transferrin reacted with Fe^{3+} in the absence of the synergistic anion carbonate and apo-transferrin reacted with Fe^{3+} in the presence of phosphate, but in the absence of carbonate. Each of these controls failed to produce the characteristic absorbance peak at 460 nm that is indicative of Fe^{3+} binding to transferrin. The results in Fig. 2 confirm that phosphate does not substitute for carbonate to produce the characteristic absorbance peak at 460 nm associated with diferric transferrin.

By confirming that phosphate does not form a ternary Fe(III)–Pi-transferrin complex that absorbs at 460 nm, we were able to test the effect of phosphate on the rate of iron loading into transferrin using the 460 nm peak [25,53]. Our goal was to determine if increasing concentrations of phosphate decreased the ability of apo-transferrin to bind Fe^{3+} to form the ternary Fe(III)–CO_3^{2}–transferrin complex. Two possible inhibitory pathways can be envisioned and are shown in Scheme 1 B & C. In the first inhibitory pathway, phosphate could compete with carbonate at the synergistic anions binding

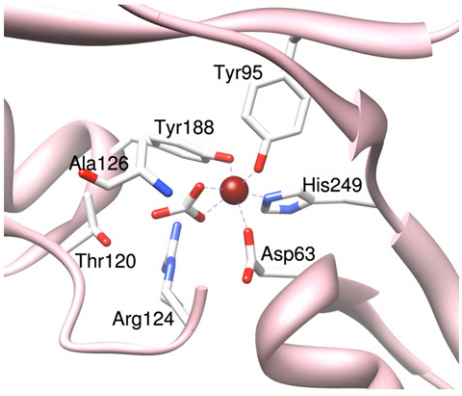

Fig. 1. Iron binding site in the N-lobe of transferrin. Four amino acid residues (Asp63, Tyr95, Tyr188, and His249) and carbonate coordinate the iron. Two residues (Arg124 and Thr 120) stabilize carbonate. PDB code 1JNF. Molecular graphics images were produced using the UCSF Chimera package from the Resource for Biocomputing, Visualization, and Informatics at the University of California, San Francisco (supported by NIH P41 RR-01081). Note that the C-lobe has a slightly different structural arrangement of ligands around the iron and carbonate [51].