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Binding of trivalent chromium to serum transferrin is sufficiently rapid to be physiologically relevant

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ABSTRACT

Transferrin, the major iron transport protein in the blood, also transports trivalent chromium *in vivo*. Recent *in vitro* studies have, however, suggested that the binding of chromic ions to apotransferrin is too slow to be biologically relevant. Nevertheless, the *in vitro* studies have generally failed to adequately take physiological bicarbonate concentrations into account. In aqueous buffer (with ambient (bi)carbonate concentrations), the binding of chromium to transferrin is too slow to be physiologically relevant, taking days to reach equilibrium with the protein's associated conformational changes. However, in the presence of 25 mM (bi)carbonate, the concentration in human blood, chromic ions bind rapidly and tightly to transferrin. Details of the kinetics of chromium binding to human serum transferrin and conalbumin (egg white transferrin) in the presence of bicarbonate and other major potential chromium ligands are described and are consistent with transferrin being the major chromic ion transporter from the blood to tissues.

1. Introduction

Transferrins are a class of proteins of ~80 kDa that reversibly bind 2 equivalents of metal ions. The protein exhibits amazing selectivity for Fe^{3+} in a biological environment because the metal sites are adapted to bind ions with large charge-to-size ratios. Transferrin, Tf, is a blood serum protein, a β-globulin, although another form of the protein is found in avian egg white, conalbumin. Transferrin is the major iron transport protein in the bloodstream, while conalbumin is believed to have antibacterial roles by depriving bacteria of iron. The transferrin molecule is composed of two lobes with approximately 40% sequence homology; the three-dimensional structures of the lobes are nearly superimposable [1]. Each lobe possesses an iron-binding site, and each Fe^{3+} binds concomitantly with a synergistic anion, usually (bi)carbonate. Fe coordination is essentially identical in each site, being distorted octahedral and comprised of two tyrosine residues, a histidine residue, an aspartate residue, and a chelating (bi)carbonate ion. The presence of the anion is essential for Fe binding. The transferrin molecule undergoes a significant conformational change when binding and releasing Fe. The apoprotein possesses a more open conformation; in the Fe-loaded conformation, transferrin binds to transferrin receptor, a transmembrane protein of the cell membrane. Transferrin is brought into the cell by endocytosis. Acidification of the resulting endosome releases the Fe^{3+}, and subsequent fusion of the endosome with the cell membrane releases and recycles the apotransferrin. In humans, the protein is present at a concentration of approximately 3 mg/mL in serum and is normally about 30% saturated with Fe, allowing it to potentially bind and transport other metal ions. For a review, see Ref. [1].

Chromium was first proposed to be an essential trace element for mammals about fifty years ago, and chromium as the trivalent ion has been accepted as an essential element for decades. That status has recently been challenged, and the status of chromium is a matter of current debate [2]. Part of the confusion with the status of Cr arises from pharmacological effects of Cr^{3+} at supranutritional doses. These doses of Cr can generate improvements in insulin sensitivity and blood cholesterol levels in animals with stresses on the glucose and lipid metabolism systems, most notably in rodent models of type 2 diabetes. For a review, see Ref. [2]. The mechanism for these effects at a molecular level is unknown; but, a role for transferrin in Cr transport associated with insulin action has been proposed.

Transferrin was first proposed to have a role in the transport and storage of chromium in the 1960s. Administration of $^{51}CrCl_3$ by stomach tube to rats resulted in ≥99% of the chromium in blood being associated with non-cellular components [3]. Ninety percent of the Cr in blood serum was associated with the β-globulin fractions, while 80% immunoprecipitated with transferrin [3]. Cr_2-transferrin serves as an inhibitor for the binding of Fe_2-transferrin to the surface of reticulocytes [4], presumably at transferrin receptor. The Cr-loaded human transferrin is a better inhibitor than apotransferrin or Cu^{2+}-loaded transferrin but not as good as mono- or diferric transferrin [4]. Injection of $^{51}Cr_2$-labeled transferrin into the bloodstream results in a rapid and insulin-sensitive movement of Cr into the tissues as Cr-transferrin [3–5];

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greater than 50% of the Cr is transported to the tissues within 30 min. Tissue levels of Cr are maximal 30 min after injection; decreases in tissue chromium with time are mirrored by increases in urine chromium. Approximately 50% of the ^{51}Cr appeared in the urine within 360 min of injection of Cr-transferrin into the tail vein of rats in the absence of added insulin; insulin treatment concurrent with injection of ^{51}Cr-labeled transferrin results in approximately 80% of the label appearing in the urine within 180 min. Therefore, transferrin, in an insulin-dependent fashion, can transfer Cr to tissues from which Cr is excreted in the urine [5–7]. Thus, transferrin appears to be the physiological transport agent for Cr^{3+}. (Although care must be taken in that when Cr^{3+} is administered intravenously or added *in vitro* to blood or blood serum or plasma, non-physiologically relevant binding of chromium to other species occurs [8–10].)

In vitro studies have shown that Cr^{3+} readily binds to the two Fe^{3+}-binding sites of apotransferrin and concomitantly also binds two equivalents of (bi)carbonate as it does when binding ferric ions [11], resulting in intense changes in the protein's ultraviolet spectrum. The amount of bicarbonate bound was determined by measuring the release of CO_2 after the addition of acid; 1.09 equivalents of CO_2 were released per bound Cr^{3+} for human transferrin [11]. The changes in the ultraviolet spectrum suggest that each chromic ion binds to two tyrosine residues, suggesting that chromium binds specifically in the two iron-binding sites; the ultraviolet absorbance maximum for human Cr_2-transferrin is at 293 nm [11]. The involvement of tyrosine ligands has been confirmed by Raman spectroscopy [12]. Human Cr_2-transferrin has been described as pale blue in color with visible maxima at 440 and 635 nm [11], while Cr_2-lactoferrin has been described as gray-green with maxima at 442 and 612 nm ($\varepsilon = 520$ and 280 $M^{-1}\ cm^{-1}$, respectively) [13]. The visible spectra are typical for Cr(III) centers in a pseudo-octahedral environment. The oxidation state of the bound chromium has been confirmed by variable temperature magnetic susceptibility studies, whose results are consistent with the presence of $S = 3/2$ centers, and by electron paramagnetic resonance (EPR) studies [11]. The two Cr-binding sites can readily be distinguished by EPR (frozen solutions at 77 K) [12]. At approximately pH 7.7, chromium binds to both sites on the protein. At pH 4.8 to 5.9, chromium binds to only one site. This tighter binding site possesses an EPR signal centered at $g = 5.43$. At near neutral pH, the Cr^{3+} in the tighter binding site that binds chromium at the lower pH cannot be displaced by Fe^{3+}, while Fe^{3+} readily displaces Cr^{3+} from the other site [12]. The weaker binding site Cr^{3+} gives rise to EPR signals at $g = 5.62$, 5.15, and 2.42 [12]. Mixed metal complexes (and their EPR spectra) with Cr^{3+} in its tight binding site and Fe^{3+} or VO_2^+ in the other binding site have been described [14]. Binding of Fe^{3+} at physiological concentrations of iron to transferrin has been reported to not be affected by a physiologically relevant concentration of Cr^{3+}; excess Fe^{3+} decreased the ability of Cr^{3+} to bind [15]. Cr^{3+} added to transferrin loaded 50% with Fe^{3+} (with the C-terminal lobe theoretically filled with Fe^{3+}) results in displacement of a few percent of the Fe^{3+} with concomitant binding of Cr^{3+} [15].

The rate at which Cr^{3+} binds to transferrin and the stability of Cr-transferrin has received considerable attention recently [15–18]. The generation of Cr_2-transferrin for the *in vitro* spectroscopic studies generally used samples of transferrin that had been allowed to come to equilibrium with Cr^{3+} ions over the course of up to two weeks to guarantee a stoichiometric amount of Cr^{3+} binding. (Additionally, questions over the extinction coefficients for the ultraviolet absorption bands used to measure Cr^{3+} binding have arisen [16].) However, whether even this leads to stoichiometric binding has been questioned in mass spectrometric studies [15,17]. This brings about an even more significant question as to how transferrin could be responsible for Cr^{3+} transport in vivo if Cr^{3+} binding actually requires days or weeks to achieve equilibrium given the half-life of transferrin in serum and that of transferrin-bound iron are in the order of hours [19,20]. Herein are reported studies on the binding of Cr^{3+} to human serum transferrin and conalbumin that reconcile these disparate results by demonstrating the importance of using carbonate concentrations that closely model physiological systems.

2. Materials and methods

2.1. Materials

Iron-free human serum transferrin and chicken conalbumin (egg white transferrin) were obtained from Aldrich (St. Louis, MO). Doubly deionized water was used throughout. All reagents were used as received unless otherwise noted. Monoferric transferrin with iron in the C-terminal metal-binding site was generated by adding Fe^{3+} as Fe(nitriloacetate)$_2$ [21]. Monoferric transferrin with iron in the N-terminal metal-binding site was generated by using ferrous ammonium sulfate by the method of Aisen and coworkers [22]. All Cr-binding studies were performed in HEPES buffer, 0.1 M, pH 7.4 in plastic centrifuge tubes. Buffer pH was adjusted by the addition of NaOH to the free acid form of HEPES. For solutions containing $KHCO_3$, pH was readjusted to 7.4 after addition of $KHCO_3$ by addition of HCl. Cr^{3+} solutions were prepared by using $CrCl_3 \cdot 6H_2O$. Co^{2+} solutions were prepared by using $CoCl_2$. Apotransferrin and apoconalbumin concentrations were determined by using the extinction coefficient ($\varepsilon = 9.12 \times 10^4\ M^{-1}\ cm^{-1}$) at 280 nm [23]. Solutions containing transferrin or conalbumin were prepared immediately before use except as otherwise noted. Similarly solutions containing added bicarbonate where prepared immediately before use. Except for the EPR spectra, all results are presented as the average of at least triplicate experiments. Error bars in figures represent standard deviation.

2.2. Instrumentation

Ultraviolet-visible spectra were obtained by using a Cary (Aligent, Santa Clara, CA) 100 or Beckman Coulter (Brea, CA) DU800 UV-visible spectrophotometer. Binding of Cr^{3+} to transferrin and conalbumin was monitored at 245 nm. Studies were initiated by the addition of Cr^{3+}. Solutions were continuously stirred by using a Starna (Atascadero, CA) "Spinette" electronic cell stirrer. Continuous wave (CW) EPR were measured on a Bruker (Billerica, MA) ELEXSYS E540 X-band spectrometer with an ER 4102 ST resonator. CW spectra were measured at 9.45 GHz with a microwave power of 8.39 mW by using a magnetic field modulation frequency of 100 kHz with an amplitude of 30 G. Spectra were taken at liquid nitrogen temperatures with a quartz insertion Dewar. To prepare EPR samples, 2 μL of the solution of the appropriate metal ion was added to 45 mL of 0.86 mM apoconablumin in 100 mM HEPES buffer, pH 7.4. After the appropriate time interval, 15 μL of glycerol was added to the samples, which were rapidly frozen in liquid nitrogen. Data analysis, calculation of averages and standard deviations and fitting of curves to the appropriate equations, was performed by using SigmaPlot 11 (SPSS, Inc., Chicago, IL). The iterative curve fitting algorithm of SigmaPlot 11 uses the Marquardt–Levenberg algorithm to find the parameters of the independent variables that provide the best fit between the data and the equation.

3. Results and discussion

3.1. Conalbumin

Given the apparent discrepancies in the ability of transferrin to serve as the physiological carrier of Cr^{3+} from the blood to the tissues and the times reported required for Cr^{3+} binding to transferrin to reach equilibrium, the binding of Cr^{3+} to conalbumin and to human serum transferrin has been re-investigated. In particular, the binding of Cr to these transferrins has been examined in the presence of varying concentrations of bicarbonate, from ambient to 25 mM, the approximate concentration in human blood plasma.