Journal of Inorganic Biochemistry 136 (2014) 24–32

Contents lists available at ScienceDirect

Journal of Inorganic Biochemistry

journal homepage: www.elsevier.com/locate/jinorgbio

Direct thermodynamic and kinetic measurements of Fe^{2+} and Zn^{2+} binding to human serum transferrin

Tyson Terpstra [a], Justin McNally [a], Thi-Hong-Lien Han [b], Nguyet-Thanh Ha-Duong [b], Jean-Michel El-Hage-Chahine [b], Fadi Bou-Abdallah [a,*]

[a] State University of New York at Potsdam, Department of Chemistry, Potsdam, NY, USA
[b] Université Paris-Diderot, Laboratoire ITODYS, Bâtiment Lavoisier, 75205 Paris, Cedex 13, France

ARTICLE INFO

Article history:
Received 16 January 2014
Received in revised form 12 March 2014
Accepted 13 March 2014
Available online 20 March 2014

Keywords:
Isothermal titration calorimetry (ITC)
Ferrous ions
Anaerobic
Transferrin
Binding thermodynamics
Kinetics

ABSTRACT

Human serum transferrin (hTf) is a single-chain bilobal glycoprotein that efficiently delivers iron to mammalian cells by endocytosis via the transferrin/transferrin receptor system. While extensive studies have been directed towards the study of ferric ion binding to hTf, ferrous ion interactions with the protein have never been firmly investigated owing to the rapid oxidation of Fe^{II} to Fe^{III} and the difficulty in maintaining a fully anaerobic environment. Here, the binding of Fe^{2+} and Zn^{2+} ions to hTf has been studied under anaerobic and aerobic conditions, respectively, in the presence and absence of bicarbonate by means of isothermal titration calorimetry (ITC) and fluorescence spectroscopy. The ITC data indicate the presence of one class of strong binding sites with dissociation constants of 25.2 nM for Fe^{2+} and 6.7 nM for Zn^{2+} and maximum binding stoichiometries of 1 Zn^{2+} (or 1 Fe^{2+}) per hTf molecule. With either metal, the binding interaction was achieved by both favorable enthalpy and entropy changes ($\Delta H^0 \sim -12$ kJ/mol and $\Delta S^0 \sim 106$ J/mol·K for Fe^{2+} and $\Delta H^0 \sim -18$ kJ/mol and $\Delta S^0 \sim 97$ J/mol·K for Zn^{2+}). The large and positive entropy values are most likely due to the change in the hydration of the protein and the metal ions upon interaction. Rapid kinetics stopped-flow fluorescence spectroscopy revealed two different complexation mechanisms with different degrees of conformational changes upon metal ion binding. Our results are discussed in terms of a plausible scenario for iron dissociation from transferrin by which the highly stable Fe^{3+}–hTf complex might be reduced to the more labile Fe^{2+} ion before iron is released to the cytosol.

1. Introduction

Transferrin and its receptor 1 is the major iron transport system in vertebrates and invertebrates [1,2]. Human serum transferrin (hTf) is a glycoprotein that belongs to the transferrin superfamily [3]. It consists of a single amino-acid chain of about 700 amino acid residues organized in two similar but not identical lobes. Each lobe contains a metal binding cleft in which Fe^{3+} is coordinated to four protein ligands: the carboxylate of an aspartate, the imidazole of a histidine and two phenolates of two tyrosines [4]. Iron is also coordinated to a synergistic anion (carbonate) without which the protein loses its high affinity for iron [5,6]. When transferrin is iron-loaded (holotransferrin), it interacts with receptor 1 and iron internalization in the cytosol occurs by receptor-mediated endocytosis [7].

Both dietary iron and recycled iron are in the Fe^{II} oxidation state [2,8]. However, the metal is transported in serum by hTf in the Fe^{III} oxidation state [8,9]. Mechanistically, iron transport from the bloodstream to the cytoplasm can be described by four successive steps: i) complex formation between serum transferrin and Fe^{3+}, ii) interaction between Fe^{3+}-loaded transferrin and receptor 1, iii) iron release (presumably in the form of Fe^{2+}) in the acidic medium of the endosome [2,9,10] and finally, iv) transfer of Fe^{2+} across the membrane of the endosome by divalent metal iron transporter proteins [2,11,12]. Up to 40 different metal ions have been shown to bind to transferrin in the presence of different synergistic anions such as bicarbonate, oxalate or nitrilotriacetate [13–18]. The fact that only about one third of the transferrin molecules in circulation is saturated with iron raises the possibility that other metal ions might bind and be transported by transferrin. Such binding may play an important role in the transport and delivery of various metal-containing compounds for diagnosis and therapy since heterometal transferrin complexes can still be recognized by the transferrin receptor. For instance, platinum complexes are used in cancer chemotherapy, gold compounds in the treatment of arthritis, gallium and indium as medical diagnostic radioisotopes, and bismuth, ruthenium and titanium are used as anti-ulcer medication [19,20 and references therein].

Recent kinetic studies have suggested an important role for the transferrin receptor in modulating the redox properties of transferrin and the subsequent mobilization of iron from the transferrin–receptor

* Corresponding author. Tel.: +1 315 267 2268; fax: +1 315 267 3170.
E-mail address: bouabdf@potsdam.edu (F. Bou-Abdallah).

http://dx.doi.org/10.1016/j.jinorgbio.2014.03.007
0162-0134/

complex [21–24]. Other studies showed that the redox potential of iron bound to the protein–receptor complex is within the range of physiological reductants [23–25] and ranges between −140 mV and −530 mV depending on the experimental conditions [26–30]. These results are consistent with mechanisms through which Fe^{III} is reduced to Fe^{II} before it is released from transferrin. Interestingly, more than two decades ago, a model for iron mobilization from transferrin has been proposed to involve intravesicular acidification followed by Fe^{3+} reduction by a membrane oxidoreductase and an Fe^{2+} transporter system for iron translocation [31]. Presumably, once inside the cell, the acidification of the endosome (down to pH ~ 5.6) weakens the affinity of iron to transferrin leading to its release [14,31,32]. Therefore, the mechanism of iron removal from transferrin at the acidic pH of the endosome is still unknown and whether reduction of Fe^{3+} bound to transferrin occurs before or after iron is released from the transferrin–transferrin receptor complex and before it is transported out of the endocytic vesicle remains an open question.

Ferrous ion is kinetically more labile than ferric ions and the affinity of hTf for Fe^{2+} is estimated to be at least 17 to 18 orders of magnitude lower than that of Fe^{3+} consistent with a reductive release of iron from transferrin [26]. The formation of metal complexes between transferrin and ferrous ions has never been measured directly because of the difficulty in maintaining a fully anaerobic environment and the rapid oxidation of Fe^{II} to Fe^{III} in the ferrous–transferrin complex [33]. However, the conditional equilibrium constants ($\log K_1 = 7.8$ and $\log K_2 = 6.4$ in 100 mM Hepes and 15 mM bicarbonate, pH 7.4 at 25 °C) for Zn^{2+} binding to apotransferrin were determined experimentally by differential UV–visible titrations using nitrilotriacetic acid and triethylenetetramine as competing ligands [34]. A linear free-energy relationship based on a series of Zn^{2+} and Fe^{2+} complexes has been constructed and on the basis of the zinc-transferrin binding constants an estimate of the Fe^{2+}–apotransferrin binding constant of $\log K_1$ of 7.4 was deduced [34]. Another study [35] has reported a linear correlation of the first metal-binding constants of human transferrin for divalent and trivalent metal ions with $\log K_1$ for the Fe^{2+}–apotransferrin complex approaching a value of 7. Here, we report for the first time a direct measurement of the binding affinity of Fe^{2+} and Zn^{2+} to human serum transferrin using isothermal titration calorimetry, spectrofluorimetric titrations and stopped-flow kinetics and discuss the mechanism of Fe^{2+} and Zn^{2+} uptake by transferrin in neutral media.

2. Materials and methods

2.1. Chemicals

Pure human serum apotransferrin (≥98%, Sigma) or lyophilized human serum apotransferrin (≥95%, CalBiochem) were either used as received or extensively dialyzed at 4 °C for 4 days. In brief, a protein sample at 10 mg/mL was initially dialyzed twice for 24 h against 0.1 M NTA and 0.1 M KCl at pH ~ 4. A second dialysis consisting of 3 changes of 8 h each against a solution of 0.2 M KCl is carried out. Finally, a third dialysis consisting of 3 changes of 8 h each was performed against a solution of 50 mM of Hepes and 130 mM KCl (pH 7.4). The molecular weight cut-off of the dialysis membrane was 50 kDa. The protein purity was checked by urea polyacrylamide gel electrophoresis and its concentration was determined spectrophotometrically using a molar extinction coefficient of 93,000 M^{-1} cm^{-1} [13,36,37]. KCl (Merck Suprapur), NaOH and HCl (Merck Titrisol), $NaHCO_3$, $Fe(NH_4)_2SO_4 \cdot 6H_2O$ and Hepes (2-[4-(2-hydroxyethyl)piperazin-1-yl] ethanesulfonic acid) (Fluka), glycerol, urea (electrophoresis reagent), SDS, Triton TX-100, ethanolamine, glycine (electrophoresis reagent) and sodium azide (Sigma), ammonium sulfate, bromophenol blue, brilliant blue, acrylamide, APS (ammonium persulfate) and TEMED (tetramethylethylenediamine) (Boehringer Mannheim), dibasic potassium phosphate trihydrate (Calbiochem) were purchased in their purest possible grade. The 3-(N-morpholino) propanesulfonic acid (Mops) buffer was obtained in the hemi-sodium salt form from Research Organics. Ferrous sulfate heptahydrate ($FeSO_4 \cdot 7H_2O$), zinc sulfate heptahydrate ($ZnSO_4 \cdot 7H_2O$), crystalline sodium chloride (NaCl) and sodium bicarbonate ($NaHCO_3$) were purchased from Fisher Scientific. The reducing agent sodium hydrosulfite (or dithionite, $Na_2S_2O_4$) was obtained at technical grade ≥85% purity from Alfa Aesar (Johnson Matthey Co.). All reagents were used as received without further purification.

2.2. Isothermal titration calorimetry

Isothermal titration calorimetry experiments were carried out on a TA Instruments low volume Nano ITC with gold cells and an active cell volume of 185 μl. All ITC titrations were performed at (25.0000 ± 0.0002) °C and a stirring rate of 250 rpm using a titrating syringe volume of 50 μl. Typically, an automated sequence of 16 injections, each of 3 μl (or 24 injections of 2 μl each) Fe^{2+} or Zn^{2+} titrant into the sample cell containing 50 or 100 μM apo-human serum transferrin, spaced at 5 min intervals to allow complete equilibration were performed with the equivalence point coming at the area midpoint of the titration. The data was collected automatically and analyzed using the NanoAnalyze software from TA Instruments and a mathematical model involving one class of independent multiple binding sites. All experiments were repeated at least five times to ensure reproducibility with a background correction control experiment using a metal ion buffered solution in the titrating syringe and a buffer solution in the ITC reaction cell to account for the heat of dilution. The errors given in the tables from curve fitting are standard errors from replicate measurements. Conditions for each ITC experiment are given in the figure captions. For more detail on the theoretical background and other practical aspects of ITC, the reader is referred to a number of excellent reviews in the literature [38–43]. The association constant (K), stoichiometry (n), the enthalpy change (ΔH^0), the Gibbs free energy ($\Delta G^0 = -RTlnK$) and the entropy change ($T\Delta S^0 = \Delta H^0 - \Delta G^0$) of the binding interaction were simultaneously determined in one single ITC experiment.

ITC experiments involving the air-sensitive Fe^{2+} ions were conducted under anaerobic conditions with a positive atmosphere of high purity argon gas (99.9995%, <5 ppm O_2). Titrant and sample solutions were made from the same stock buffer solution of either 50 or 100 mM Mops, 50 mM NaCl, pH 7.01 or 7.45 and thoroughly deoxygenated before each titration using a degassing station provided by TA Instruments (TA Model 6326). In these anaerobic experiments, 10 mM dithionite ($Na_2S_2O_4$) was added to the protein and the Fe^{2+} solutions as a precaution to prevent oxidation from possible residual O_2.

2.3. Spectrofluorimetric measurements

Fluorimetric measurements were performed at (25.0 ± 0.1) °C and pH 7.0 on a Fluorolog 3 Horiba Jobin-Yvon fluorimeter equipped with external thermostated water-bath circulation. The excitation wavelength was set at 280 nm and the emission spectra were collected between 300 and 400 nm. The apoprotein concentrations were 0.083 and 0.084 μM with Zn^{2+} and Fe^{2+} ion concentrations varying between 0 μM to 0.71 μM and 0 μM to 2.01 μM corresponding to ratios of 0–8.5 and 0–24 Zn^{2+} and Fe^{2+} ions per apoprotein, respectively. Several metal ion stock solutions have been prepared (i.e. $[Fe^{2+}] = 5$, 10 and 50 μM; $[Zn^{2+}] = 0.1$, 0.5, 1, 10 and 50 μM) and the volumes of the fluorescence cells used in these spectrofluorimetric titrations were 3 mL for Zn^{2+} and 1 mL for Fe^{2+}. Typically, 16 injections of 1 to 10 μl of Fe^{2+} and 24 injections of 1 to 10 μl of Zn^{2+} were made to the same protein sample and a volume correction was applied in our data analysis. The light path length was 1 cm and the monochromator slit openings were 2 nm and 4 nm for excitation and emission, respectively. The spectra used for the determination of equilibrium constants were recorded at the final equilibrated state of the species involved. For the anaerobic experiments, the especially designed cuvettes (100 or 1000 μl reaction volume) and all solutions were prepared and kept in a glove box