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Iron uptake and transfer from ceruloplasmin to transferrin

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ABSTRACT

Background: Dietary and recycled iron are in the Fe^{2+} oxidation state. However, the metal is transported in serum by transferrin as Fe^{3+}. The multi-copper ferroxidase ceruloplasmin is suspected to be the missing link between acquired Fe^{2+} and transported Fe^{3+}.
Methods: This study uses the techniques of chemical relaxation and spectrophotometric detection.
Results: Under anaerobic conditions, ceruloplasmin captures and oxidizes two Fe^{2+}. The first uptake occurs in domain 6 (<1 ms) at the divalent iron-binding site. It is accompanied by Fe^{2+} oxidation by Cu^{2+}_{D6}. Fe^{3+} is then transferred from the binding site to the holding site. Cu^{+}_{D6} is then re-oxidized by a Cu^{2+} of the trinuclear cluster in about 200 ms. The second Fe^{2+} uptake and oxidation involve domain 4 and are under the kinetic control of a 200 s change in the protein conformation. With transferrin and in the formed ceruloplasmin–transferrin adduct, two Fe^{3+} are transferred from their holding sites to two C-lobes of two transferrins. The first transfer (~100 s) is followed by conformation changes (500 s) leading to the release of monoferric transferrin. The second transfer occurs in two steps in the 1000–10,000 second range.
Conclusion: Fe^{3+} is transferred after Fe^{2+} uptake and oxidation by ceruloplasmin to the C-lobe of transferrin in a protein–protein adduct. This adduct is in a permanent state of equilibrium with all the metal-free or bounded ceruloplasmin and transferrin species present in the medium.
General significance: Ceruloplasmin is a go-between dietary or recycled Fe^{2+} and transferrin transported Fe^{3+}.

1. Introduction

Both dietary and recycled iron are in the Fe^{2+} oxidation state [1,2]. However, the metal is transported in serum by the iron-transport protein, transferrin (T) in the Fe^{3+} oxidation state [1,3]. This implies the existence of a mediator capable of taking charge of the acquired and recycled Fe^{2+} in order to deliver it as Fe^{3+} to transferrin. During this process, iron, in its two oxidation states, should never be allowed to enter into contact with the aqueous biological environment. Indeed, both oxidation states are involved in Fenton and Haber–Weiss chemistries. These are responsible for generating highly reactive oxygen radicals that cause irreversible damage to cells and their environment [2,4,5]. Ceruloplasmin (Cp) is strongly suspected of being this mediator which can play the role of an antioxidant inhibiting free radical formation [6–9]. Another hypothesis envisages a Fe^{2+} capture by T and its oxidation to Fe^{3+} once bound because of the strongly negative redox potential (−500 mV at pH 5.6) for iron in the protein [10].

Human serum ceruloplasmin (Cp) is a multifunctional glycoprotein that carries 95% of the plasma copper in mammals [11]. This protein oxidizes a number of structurally unrelated molecules, such as 4-phenylenediamine, amino-phenols, catechols and 5-hydroxyindoles and even nitric oxide [12–15]. However, its main activity is assumed to be that of ferroxidase. This assumption is confirmed by the facts that humans with mutations of the ceruloplasmin gene (aceruloplasminemia) show iron accumulation in several organs including the retina, liver and brain [16]. This loss of the ferroxidase activity leads to the accumulation of an excess of iron in the liver, spleen and pancreas of pigs and rodents, as well as in ceruloplasmin knockout mice [17–21]. Cp is a 132 kDa enzyme that consists of a single polypeptide chain of 1046 amino acids folded in six domains [22–24]. It is a member of the multi-copper oxidase family that comprises ascorbate oxidase and the laccases [25]. These enzymes are characterized by three types of copper-binding sites [26]. In Cp there are three type 1 coppers, which are responsible for its typical blue color, with an absorption maximum, $\lambda_{Cp} = 610$ nm. The type 1 occupies a distorted tetrahedral site which is shaped by two histidines, one cysteine along with one methionine, or more rarely, a leucine [27]. Furthermore, three other coppers are engaged in a cluster, in which a pair of type 3 copper atoms are attached to a type 2 site [28]. The trinuclear coordination site consists of four pairs of histidine. The two type 3 (T3) copper are bound to six histidines and constitute a pair of antiferromagnetically coupled copper ions, whereas the type 2 (T2) copper is coordinated by two histidines. A dioxygen is bound to the trinuclear copper cluster [29].

Abbreviations: Cp, ceruloplasmin; T, apotransferrin; Cp-T, ceruloplasmin–transferrin protein–protein adduct; Cu_{D6}, type 1 copper of domain 6 in Cp; Cu_T, type 3 copper of the trinuclear cluster of Cp; Cu_{D4}, type 1 copper of domain 4; TFe_2, holotransferrin; T_CFe, $T_CFe\text{-}T_N$, TFe^{3+} C-lobe monoferric transferrin; GSH, reduced glutathione

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The cluster is sufficiently close to the type 1 (T1) coppers to allow an electron transfer between the latter and the trinuclear center. This provides for the reduction of the dioxygen and the release of two water molecules without any liberation of reactive oxygen species [30,31].

Transferrin is a glycoprotein of 700 amino-acids organized in two semi-equivalent lobes, each of which contains an iron-binding site composed of two phenols of two tyrosines, a carboxylate of an aspartate, an imidazole of a histidine and a synergistic carbonate adjacent to an arginine. The apo-form (iron-free) is in the so-called open conformation where the protein ligands are in direct contact with the bulk medium. In the iron-loaded transferrin or holotransferrin (TFe_2), each lobe encloses the coordinated metal, which becomes buried about 10 Å under the surface of the protein in a closed conformation [32–34]. T has a very high affinity for Fe^{3+} (10^{21}) and a much lower one for Fe^{2+} (~10^7) [35,36]. TFe_2 interacts extremely rapidly with transferrin receptor 1, which is anchored in the plasma membrane [37]. The two proteins in interaction are then internalized in the cytoplasm by receptor-mediated endocytosis [38].

Fe^{3+} is basically insoluble in aqueous neutral media, where it readily precipitates as different iron hydroxide species. Fe^{3+} is usually solubilized in the form of low-molecular-mass chelates or complexed to proteins or macromolecules. In the normal individual about 0.1% of the total body iron (i.e. about 3 to 4 mg) is in the plasma, bound predominantly to transferrin (which is approximately 30% saturated with iron). The daily turnover of iron is about 30 mg. Thus, transferrin is continually binding iron (both dietary and recycled iron) and delivering it to cells and tissues [2,3,39,40]. We established a few years ago the mechanisms of Fe^{3+} uptake by transferrin from soluble complexes, and showed that these processes occur in several kinetic steps ranging from milliseconds to hours [41,42]. The concentration of non-transferrin-bound iron in the serum (≤0.1 mg) is extremely low [2,43,44]. This, added to the rates we reported for Fe^{3+} uptake by transferrin from chelates, is far from sufficient for the required iron turnover. How, then, is iron acquired by transferrin, and is Cp involved?

We recently showed that Cp interacts with both apo- and holotransferrin with a dissociation constant of about 15 μM [45]. These interactions are extremely weak as compared to that of TFe_2 or the C-lobe-only iron-loaded transferrin ($T_CFe\text{-}T_N$) with receptor 1 (2.3 nM) [37,46]. Cp cannot, therefore, interfere with the recognition of TFe_2 by the receptor. In the blood stream the concentrations of Cp (2.5 μM) and transferrin (25 μM) [47,48], imply that 70% of the circulating Cp is interacting with transferrin [45]. However, this does not provide any evidence for a transfer of Fe^{3+} from Cp to T.

The work presented here consists of two essential parts. In the first, we analyze Fe^{2+} uptake by Cp and revisit its oxidation to Fe^{3+}. In the second, we research the possibility of an intramolecular Fe^{3+} transfer from Cp to T in the Cp–T protein–protein adduct. These investigations are based on the use of chemical relaxation and spectrophotometric techniques [49,50].

2. Material and methods

2.1. Material

Apotransferrin was purchased from Sigma, and purified according to published procedures [41]. Ceruloplasmin was purchased from Euromedex, and used without further purification. The copper load was checked as described elsewhere [51]. Commercial ceruloplasmin carries 5 Cu^{2+} and 2 Cu^{+} and is iron-free. KCl (Merck Suprapur), NaOH, HCl (Merck Titrisol), $NaHCO_3$, HEPES (2-[4-(2-hydroxyethyl)piperazin-1-yl] ethanesulfonic acid), sodium ascorbate, $Fe(NH_4)_2SO_4 \cdot 6H_2O$ (Fluka) and reduced glutathione (Sigma) were of the purest possible grades.

2.1.1. Stock solutions

The HEPES concentration in neutral buffers was 50 mM. Final pHs were continuously controlled and adjusted to between 6.6 and 8.6 with micro-quantities of concentrated HCl or NaOH. Cp concentrations (c_1) were checked by a Bio Rad protein assay and spectrophotometrically [52]. Final solutions of Cp and T (c_2) were diluted further to the required concentrations in the buffers. c_1 varied from 50 nM to 5 μM and Fe^{2+} concentrations (c_3) from 15 to 1000 μM. All final ionic strengths were adjusted to 0.2 M with KCl.

Crystalline ferrous ammonium sulfate hexahydrate, $Fe(NH_4)_2SO_4 \cdot 6H_2O$, was used as the ferrous iron source and was dissolved at 10 mM in a 0.2 M deoxygenated KCl solution. Ferrozine (3-(2-pyridyl)-5,6-bis(4-phenylsulfonic acid)-1,2,4-triazine) was dissolved at 2.5 mM in 0.1 M NaCl. Sodium ascorbate ((2*R*)-2-[(1*S*)-1,2-dihydroxyethyl]-4-hydroxy-5-oxo-2*H*-furan-3-olate) and reduced glutathione were dissolved in deoxygenated 50 mM Hepes, 20 mM $NaHCO_3$, and 130 mM KCl buffer. Q3

2.2. Methods

2.2.1. pH measurements

pH values were measured in a glove box with a Jenco pH-meter equipped with a "Metrohm" combined calomel/glass mini-electrode. The pH-meter was standardized at working temperature by standard buffers: pH 7.00 and 10.01 (Beckman). At the end of the measurements, pHs were double-checked both in the buffer and in the protein solutions. They were accurate to better than 0.02 pH unit.

2.2.2. Spectrophotometric measurements

Absorption measurements were performed at (25 ± 0.1) or (10 ± 0.1) °C on a Cary 4000 spectrophotometer equipped with a Peltier thermostated cell-carrier. Fluorimetric measurements were performed at (25 ± 0.1) or (10 ± 0.1) °C on a Fluorolog 3 Horriba Jobin-Yvon equipped with a thermostated cell-carrier. Emission spectra were measured in the 300–400 nm range. Excitation wavelength was set to 280 nm. The spectra used for equilibrium constants determinations were recorded at the final equilibrated state. The cuvettes had a capacity of 100 or 1000 μL. They were especially designed to be used under anaerobic conditions and were, when required, manipulated in a glove box before being transferred to the spectrometers (transfer time about 2 min).

2.2.3. Ferroxidase activity

Ferroxidase activity was measured with ferrous ammonium sulfate as substrate, in 50 mM HEPES, 20 mM $NaHCO_3$, 130 mM KCl, pH between 6.6 and 8.5 at 10 and 25 °C. Cp (50 nM) was incubated with ferrous iron solution (10^{-7}–10^{-3} M). Aliquots were removed at specific time intervals and added to 2.5 mM of ferrozine solution, which stopped the enzymatic reaction and allowed the detection of the ferrous-ferrozine complex ($\varepsilon_{562} = 27\,900\ M^{-1}\ cm^{-1}$) [53].

2.2.4. Dissociation constants of Cp–Fe complexes

Dissociation constants were determined spectrophotometrically by the use of the Global Analysis program SPECFIT 32. SPECFIT is a multivariate data analysis program for data sets that are obtained from multiwavelength spetrophotometric measurements. The program utilizes a specially adapted version of the Levenberg–Marquardt method. This procedure returns optimized model parameters, their standard errors, and the predicted spectra of the unknown colored species [54]. The dissociation constants were checked when possible by a variant of the Benesi and Hildebrand method [45].

2.2.5. Kinetics

Stopped-flow experiments were performed under anaerobic conditions by mixing solutions of ceruloplasmin or ceruloplasmin–apotransferrin with Fe^{2+} on a Hi-Tech Scientific SF61DX2 stopped-flow spectrophotometer equipped with a Xe/Hg light source and a thermostated bath held at 10 or 25 °C. The mixing syringes, reservoirs and chambers were kept under argon in a specially designed box. The solutions were prepared in a glove box and introduced by syringes kept under argon in

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