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Neither Human Hephaestin Nor Ceruloplasmin Forms a Stable Complex With Transferrin

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Abstract Iron homeostasis is essential for maintaining the physiological requirement for iron while preventing iron overload. Cell toxicity is caused by the generation of hydroxyl-free radicals that result from redox reactions involving Fe(II). Multicopper ferroxidases regulate the oxidation of Fe(II) to Fe(III), circumventing the generation of these harmful by-products. Ceruloplasmin (Cp) is the major multicopper ferroxidase in blood; however, hephaestin (Hp), a membrane-bound Cp homolog, was recently discovered and has been implicated in the export of iron from duodenal enterocytes into blood. In the intracellular milieu, it is likely that iron exists as reduced Fe(II), yet transferrin (Tf), the plasma iron transporter, is only capable of binding oxidized Fe(III). Due to the insoluble and reactive nature of free Fe(III), the oxidation of Fe(II) upon exiting the duodenal enterocyte may require an interaction between a ferroxidase and the iron transporter. As such, it has been suggested that as a means of preventing the release of unbound Fe(III), a direct protein–protein interaction may occur between Tf and Hp during intestinal iron export. In the present study, the putative interaction between Tf and both Cp and a soluble form of recombinant human Hp was investigated. Utilizing native polyacrylamide gel electrophoresis, covalent cross-linking and surface plasmon resonance (SPR), a stable interaction between the two proteins was not detected. We conclude that a stable complex between these ferroxidases and Tf does not occur under the experimental conditions used. We suggest alternative models for loading Tf with Fe(III) during intestinal iron export. J. Cell. Biochem. 103: 1849–1855, 2008.

Key words: hephaestin; ceruloplasmin; transferrin; ferroxidase; iron; surface plasmon resonance

The dietary absorption of iron, its transport in blood, and subsequent storage in peripheral tissues are all vital but incompletely understood steps in human iron homeostasis. In the gut, dietary Fe(III) is reduced to Fe(II) by the ferrireductase duodenal cytochrome B and transported into the duodenal enterocyte via DMT1 [Mackenzie and Garrick, 2005]. At the basolateral membrane, Fe(II) is exported into the extracellular environment via the transmembrane permease ferroportin [Mackenzie and Garrick, 2005]. Outside the cell, Fe(II) is oxidized to Fe(III) prior to loading onto serum transferrin (Tf), the iron carrier in blood. Tf specifically binds Fe(III), but not Fe(II), very tightly with a K_D of $\sim 10^{-24}$ M at physiological pH [Richardson and Ponka, 1997]. Iron-loaded Tf (holo-Tf) can then travel throughout the circulation and deliver Fe(III) to all tissues via a binding interaction with the transferrin receptor (TfR). Prior to binding to Tf, the oxidation of Fe(II) to Fe(III) is thought to be facilitated by a ferroxidase protein [Aisen et al., 2001].

Ceruloplasmin (Cp) plays a vital role in mammalian iron homeostasis as the major multicopper ferroxidase in blood [Hellman and

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Gitlin, 2002]. Deficiencies in Cp result in iron accumulation in cells that can lead to progressive neurodegenerative disease [Harris et al., 1995]. Iron release from non-intestinal cells requires Cp ferroxidase activity, yet it is not known how Fe(III) oxidized by Cp is transferred to Tf in the serum [Hellman and Gitlin, 2002]. Hephaestin (Hp), a homolog of Cp, is a membrane-bound multicopper ferroxidase [Griffiths et al., 2005] that has been implicated in the export of iron from duodenal enterocytes into the circulation [Li et al., 2003]. Sex-linked anemia, a condition that results in severe iron deficiency in mice, was first identified as a 582 base pair deletion in the *hephaestin* gene [Vulpe et al., 1999]. Since its discovery, Hp has been localized in many different tissues including the small intestine [Frazer et al., 2001], colon [Brookes et al., 2006], retinal pigment epithelial [Hahn et al., 2004], heart [Qian et al., 2007a], and brain [Qian et al., 2007b]. In the duodenum, Hp has been localized to both the basolateral membrane and the apical supranuclear membrane of the enterocyte and is a likely candidate for the function of loading Tf with Fe(III) [Simovich et al., 2002; Kuo et al., 2004].

Due to the insoluble nature of free Fe(III) under physiological conditions and the potential for both Fe(II) and Fe(III) to generate harmful hydroxyl-free radicals, the regulation of iron is fundamental to the prevention of cell toxicity [Aisen et al., 2001]. It has been suggested that as a means of preventing the release of unbound Fe(III), a direct protein–protein interaction may occur between Tf and Hp during intestinal iron export [Syed et al., 2002; Griffiths et al., 2005]. This hypothesis is supported by a recent study that reported the formation of a stable complex between a multicopper ferroxidase and Tf in algae [Paz et al., 2007]. Furthermore, the identification of an interaction between Cp and lactoferrin (a Tf analog) under physiological conditions provides additional support [Pulina et al., 2002; Sokolov et al., 2006]. However, there have been no reports to date that confirm an interaction between serum Tf and any human multicopper ferroxidases.

Previous work in this laboratory has shown that the loading of Tf with Fe(II) was enhanced by a soluble form of recombinant human Hp (rHp) [Griffiths et al., 2005]. This was consistent with the specificity of Tf for Fe(III) and also suggested a potential interaction between the two proteins. In the present study, the putative interaction between Tf and both rHp and Cp was investigated. Utilizing native polyacrylamide gel electrophoresis (PAGE) and covalent chemical cross-linking, all attempts to identify a stable protein–protein complex proved negative. To study a more transient interaction between the two proteins, surface plasmon resonance (SPR) was employed using a variety of conditions; again, no detectable interaction was observed. We conclude that the human ferroxidases and Tf do not interact directly and suggest alternative mechanisms for iron-loading Tf during intestinal iron export.

MATERIALS AND METHODS

Proteins

Soluble human rHp was expressed as described previously [Griffiths et al., 2005]. Briefly, rHp was engineered to express a soluble protein by replacing the C-terminal transmembrane domain of rHp with a cleavable 1D4 epitope tag. The construct was cloned into a pNUT expression vector and expressed in baby hamster kidney cells. Human Cp was purchased from VitalProducts, Inc., (Boynton Beach, FL). Human apotransferrin (apo-Tf) and holo-Tf were purchased from Sigma-Aldrich (Oakville, ON). Soluble TfR (sTfR), expressed as previously described [Byrne et al., 2006], was kindly provided by Dr. Anne Mason (Department of Biochemistry, University of Vermont). Anti-1D4 antibody was kindly provided by Dr. Robert Molday (Department of Biochemistry and Molecular Biology, University of British Columbia).

Ferroxidase Activity

Oxidation of Fe(II) by rHp was determined using a ferrozine-based ferroxidase assay as described previously [Reilly and Aust, 1997]. Briefly, rHp was incubated with 100 M equivalents of Fe(II):imidazole (1:5) in HBS (HEPES-buffered saline, 0.01 M HEPES pH 7.4 containing 0.15 M NaCl) in the presence or absence of apo-Tf. Ferrozine quenches the ferroxidase reaction by binding residual Fe(II), the ferrous–ferrozine complex was measured spectrophotometrically ($\varepsilon_{562} = 27900\ M^{-1}cm^{-1}$). Autoxidation was determined in the absence of rHp and Tf. All assays were performed in triplicate.

Gel Electrophoresis

Native PAGE used 5% polyacrylamide gels in the absence of detergent, reducing agent and sample heating. Protein samples were incubated at room temperature for 30 min in HBS prior to being electrophoresed at 5 mA. All other gel analyses utilized 7.5% polyacrylamide gels containing 1% sodium dodecyl sulphate (SDS) and 125 mM dithiothreitol [Griffiths et al., 2005]. Gels were stained with EZBlue™ Coomassie Brilliant Blue G-250 (Sigma-Aldrich).

Covalent Chemical Cross-Linking

Optimal chemical cross-linking conditions were determined empirically using the homo-bifunctional chemical cross-linking reagent bis(sulfosuccinimidyl)suberate (BS^3). Proteins (5 μM) were incubated in HBS at room temperature for 15 min prior to being treated with 5 mM BS^3 (Pierce Chemical Company, Rockford, IL) and an additional incubation for 2 h at room temperature. Untreated samples were prepared in the absence of BS^3.

Surface Plasmon Resonance

Potential interactions with apo-Tf and holo-Tf were investigated using a Biacore 3000 (Biacore AB, Uppsala, Sweden). The immobilized ligands were rHp, Cp, and sTfR. Proteins (rHp and Cp, 150 μg/ml) were immobilized on a CM5 sensor chip in random orientation (Biacore AB) using amine-coupling chemistry to 7000–8500 response units (RU). Apo-Tf and holo-Tf were injected over the rHp flow cell at 24°C in HBS with a 5 min association phase and a 6 min dissociation phase and the Cp flow cell with an association time of 2 min. As a control, the interaction between rHp (containing a C-terminal 1D4 epitope) and the anti-1D4 antibody was performed. The antibody titration series was analyzed with association and dissociation phases of 6 min each. A regeneration phase of 30 min with 1.2 μg/μl 1D4 peptide followed each cycle in the antibody titration series. As a further control, the interaction of sTfR with holo-Tf was analyzed as described [Lebron et al., 1998]. sTfR was immobilized to a CM5 sensor chip in random orientation to 1900 RUs. Data collection for association phase was 4 min with a 6 min dissociation phase in HBS containing 0.005% P-20 detergent. All experiments used a flow rate of 10 μl/min. A blank flow cell was used as a reference for all the data. Dissociation constants were determined using GraphPad Prism 4.1 (GraphPad Software, San Diego, CA).

RESULTS

Ferroxidase Activity

Ferroxidase activity was confirmed using an assay in which rHp oxidized Fe(II) to Fe(III) (Fig. 1). It has previously been shown that apoferritin, but not apo-Tf, is able to increase the ferroxidase activity of Cp due to a direct interaction [Reilly and Aust, 1997; Reilly et al., 1998]. Neither apo-Tf (Fig. 1) nor ferritin (data not shown) was able to enhance the ferroxidase activity of rHp, suggesting the absence of a similar interaction. This suggested that Tf was not acting as sink for released Fe(III). Under the conditions used, autoxidation from atmospheric oxygen tension or Tf was negligible.

Gel Electrophoresis

Native PAGE was used in the initial investigation to detect protein complexes (Fig. 2). A complex of proteins would be expected to migrate separately from the individual proteins. This was observed with the sTfR + holo-Tf complex (lane 6), which migrated between both of the individually electrophoresed proteins (lanes 2 and 3). No distinct molecular weight species were detected in the mixed sample of rHp and apo-Tf (lane 8), or Cp and apo-Tf (lane 7) compared to the individual proteins (lanes 1, 4, and 5) suggesting the absence of any stable protein–protein complexes.

The possibility that a complex could be formed in solution but dissociate during the electrophoresis was also tested. The homo-bifunctional

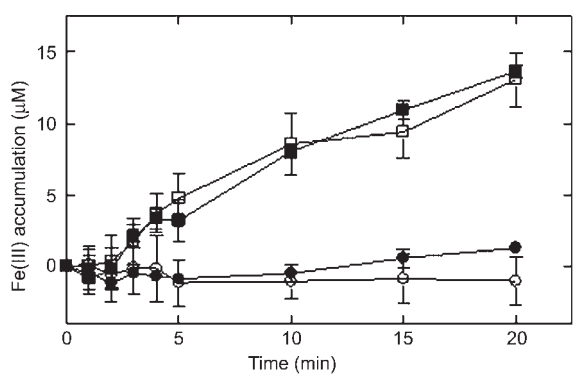

Fig. 1. Ferroxidase activity of rHp in the presence and absence of Tf using a ferrozine-based ferroxidase assay. Hp (■), Hp and Tf (□), Tf (●), autoxidation in the absence of added proteins (○). Data represent the mean ± standard deviation.

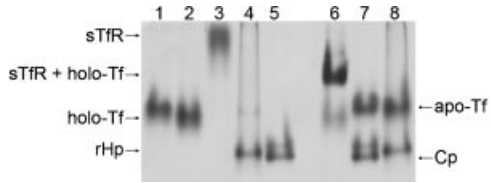

Fig. 2. Putative complex formation between rHp and Tf analyzed by 5% native PAGE. Proteins (10 μg) were prepared in the absence of detergent, reducing agent and heating. **Lane 1**, apo-Tf; **lane 2**, holo-Tf; **lane 3**, sTfR; **lane 4**, rHp; **lane 5**, Cp; **lane 6**, sTfR and holo-Tf; **lane 7**, Cp and apo-Tf; **lane 8**, rHp and apo-Tf.

cross-linking reagent BS^3 was used to covalently join any short-lived complexes in solution. Initial evaluation revealed that BS^3 was able to capture homodimers of both sTfR $(sTfR)_2$ and rHp $(rHp)_2$ (Fig. 3; lanes 4 and 10). This result is expected for the sTfR as it is known to exist as a disulfide-linked dimer. However, it is not known if rHp oligomerizes in nature, making the presence of the $(rHp)_2$ dimer difficult to evaluate. A high molecular weight band was observed with the sTfR + holo-Tf cross-linked complex (lane 1), that was absent in the individually cross-linked and untreated samples (lanes 2–5). The absence of a higher molecular weight species in the BS^3-treated samples of rHp and apo-Tf (lane 6) and Cp and apo-Tf (lane 7) compared to the controls (lanes 8–13) strongly suggests that the ferroxidases do not form a complex with Tf in vitro. Similar cross-linking experiments using the long-chain heterobifunctional cross-linker sulfo-KMUS (Pierce) also failed to show any interactions (data not shown).

Surface Plasmon Resonance

SPR analysis was used to determine whether rHp or Cp was involved in a transient interaction with Tf in a real-time analysis. No response was observed in the rHp-bound flow cell with Tf concentrations up to 30 μM (using apo- and holo-Tf; Fig. 4A). No interaction was found between Cp and Tf under similar conditions (Fig. 4C). Immobilization of rHp in a fixed orientation using a 1D4 antibody capture method also failed to show an interaction (data not shown). This provided further evidence that these multicopper ferroxidases do not participate in a direct protein–protein interaction with Tf. The presence of rHp on the biosensor chip was verified by injecting anti-1D4 antibody under identical conditions as injected Tf ($K_D = 134 \pm 30$ nM; Fig. 4A). Comparatively, holo-Tf bound to immobilized sTfR at concentrations as low as 0.25 nM. Near steady-state analysis of holo-Tf binding to sTfR resulted in two dissociation constants when fitted to a bivalent ligand (2:1) model. Each sTfR was determined to be able to bind two holo-Tf molecules in a stepwise manner with $K_{D1} = 10.7 \pm 1.2$ nM and $K_{D2} = 158 \pm 39$ nM (Fig. 4B). These values are in reasonable agreement with previous studies using other preparations of recombinant, soluble TfR ($K_{D1} \sim 3$ nM and $K_{D2} \sim 29$ nM) [Lebron et al., 1998] and membrane-bound TfR ($K_D \sim 7$ nM) [Dautry-Varsat et al., 1983].

DISCUSSION

To investigate a potential interaction with Tf, a soluble form of the Hp ectodomain was expressed with a C-terminal 1D4 epitope tag. All assays were designed to facilitate the comparison of a Tf interaction with each rHp, Cp, and sTfR. sTfR was used as a positive control because TfR is the known physiological receptor for Tf. Furthermore, both rHp and sTfR

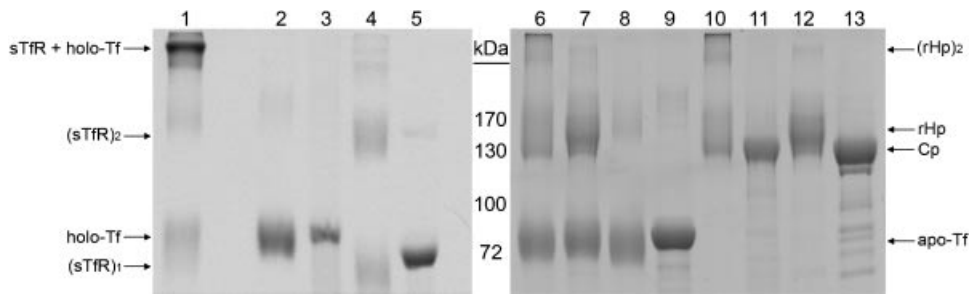

Fig. 3. Analysis of putative protein–protein interactions using chemical cross-linking. After cross-linking with BS^3 protein species were analyzed on 7.5% SDS–PAGE. **Lane 1**, sTfR + holo-Tf + BS^3; **lane 2**, holo-Tf + BS^3; **lane 3**, untreated holo-Tf; **lane 4**, sTfR + BS^3; **lane 5**, untreated sTfR; **lane 6**, rHp + apo-Tf + BS^3; **lane 7**, Cp + apo-Tf + BS^3; **lane 8**, apo-Tf + BS^3; **lane 9**, untreated apo-Tf; **lane 10**, rHp + BS^3; **lane 11**, untreated rHp; **lane 12**, Cp + BS^3; **lane 13**, Cp untreated.

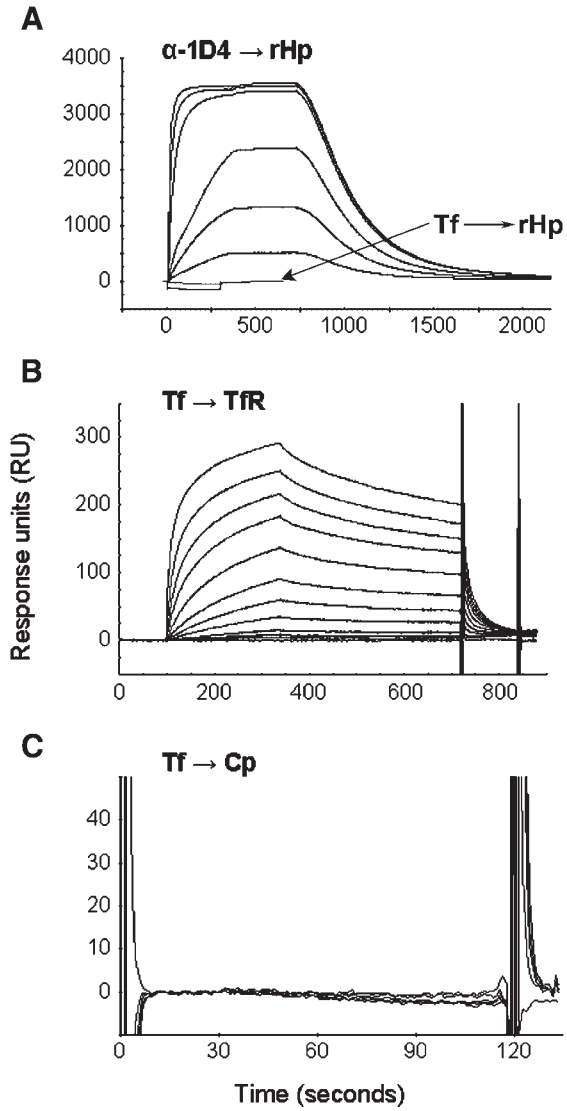

Fig. 4. SPR analysis of Tf binding to immobilized rHp, sTfR, and Cp. **A**: Binding profiles of apo- and holo-Tf (3.0 and 30 μM; indicated by arrow) and anti-1D4 antibody (5.0, 10, 25, 50, 100, 200 ng/μl) to immobilized rHp. **B**: Binding profiles of holo-Tf (0, 0.25, 0.5, 1.0, 2.5, 5.0, 10, 25, 50, 100, 200, 500 nM) to immobilized sTfR. **C**: Binding profiles of apo- and holo-Tf (0, 3.0, and 30 μM) to immobilized Cp.

are membrane-bound proteins that have been expressed as soluble recombinant proteins using analogous expression systems.

Multi-copper ferroxidases such as Hp and Cp provide the catalysis for the oxidation of Fe(II) to Fe(III) and are essential for the mobilization of intracellular iron into the extracellular environment [Vulpe et al., 1999]. As a means of preventing potential damage caused by unbound Fe(III), it has been suggested that Tf and Hp may function together in the export of iron out of the duodenal enterocyte [Syed et al., 2002; Griffiths et al., 2005]. In the present study, it was established that ferroxidases Hp and Cp do not form a stable complex or directly interact with Tf in solution. This was confirmed using a suite of techniques, including gel analysis and SPR.

Cp and Hp are paralogous proteins that have non-identical functions in iron metabolism [Chen et al., 2004]. Although Hp is known to function as a ferroxidase [Frazer et al., 2001; Griffiths et al., 2005], its specific role in iron metabolism remains unclear. The localization of Hp to both the intracellular and extracellular environments presents another element of uncertainty concerning its function, as it is not clear how an intracellular ferroxidase would function in iron transport. A recent study, however, has implicated an intracellular Cp as well as Hp as having roles in iron absorption [Cherukuri et al., 2005]. As well, both DMT1 and apo-Tf have also recently been localized to the supranuclear compartment in an intestinal cell line [Ma et al., 2002], supporting the possibility of a role for an intracellular ferroxidase in vesicular transport or transcytosis. The processing of absorbed iron by Hp or Cp inside the cell could also explain the absence of a direct interaction with Tf observed in this study.

Whether Tf actually plays a significant role in iron absorption must also be considered in this interaction study. It has been suggested that during iron absorption from the enterocyte, the levels of free iron released exceed the binding capacity of Tf [Morgan, 1980]. Furthermore, in separate studies using hypotransferrinemic mice as a model, it was proposed that Tf may not be required for intestinal iron absorption [Crazen et al., 1987; Raja et al., 1999]. Another explanation for the lack of interaction between the ferroxidase and Tf is based on their physical locations; upon oxidation, Fe(III) would be released into the mucosa, while Tf is typically found circulating throughout the vasculature. In this situation, an unknown iron carrying intermediate, such as citrate, could transport iron through the interstitial fluid to portal blood; thereby bypassing the need for a direct ferroxidase–Tf interaction. On the other hand, with Tf serum concentrations as high as ~50 μM [Richardson and Ponka, 1997], Tf may act as an efficient scavenger for released Fe(III) without the requirement for intermediates or direct protein–protein interactions.

Finally, it is possible that Tf is only capable of recognizing membrane-bound Hp if ferroportin is present, rather than the soluble ferroxidase alone (used in the current studies). Indirect evidence for an interaction between Hp and ferroportin arose from studies of the yeast Hp homolog Fet3p and the iron transporter Ftr1p [Bonaccorsi di Patti et al., 2005; Kwok et al., 2006]. Ferroportin and Hp have also been shown to colocalize on the basolateral membrane of human intestinal absorptive cells [Han and Kim, 2007]. Furthermore, ferroportin has been shown to export iron both in the presence of Tf but the absence of ferroxidase [Donovan et al., 2000] and the presence of ferroxidase but the absence of Tf [McKie et al., 2000]. Together, these data suggest that there is a complex network of interactions between these proteins that remains to be resolved. Further work is required to elucidate the role of each of these proteins in the process of duodenal iron absorption. Additional studies are also needed to clarify the localization and function of Hp.

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