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In Vitro Interaction between Ceruloplasmin and Human Serum Transferrin

Nguyêt-Thanh Ha-Duong,* Chantal Eid, Miryana Hémadi, and Jean-Michel El Hage Chahine*

Laboratoire ITODYS (Interfaces, Traitements et Organisation des Systèmes), Université Paris-Diderot, CNRS UMR 7086, Bâtiment Lavoisier, 15 rue Jean-Antoine de Baïf, 75205 Paris Cedex 13, France

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ABSTRACT: The thermodynamics of the interactions of serum apotransferrin (T) and holotransferrin (TFe_2) with ceruloplasmin (Cp), as well as those of human lactoferrin (Lf), were assessed by fluorescence emission spectroscopy. Cp interacts with two Lf molecules. The first interaction depends on pH and μ, whereas the second does not. Dissociation constants were as follows: $K_{11Lf} = 1.5 \pm 0.2\ \mu M$, and $K_{12Lf} = 11 \pm 2\ \mu M$. Two slightly different interactions of T or TFe_2 with Cp are detected for the first time. They are both independent of pH and μ and occur with 1:1 stoichiometry: $K_{1T} = 19 \pm 7\ \mu M$, and $K_{1TFe2} = 12 \pm 4\ \mu M$. These results can improve our understanding of the probable process of the transfer of iron from Cp to T in iron and copper transport and homeostasis.

Ceruloplasmin (Cp) is a protein that circulates in the blood and plays a major role in iron and copper homeostasis (*1*, *2*). More than 95% of the copper in the plasma is bound to Cp, which also possesses two iron binding sites (*3*). Cp is in addition one of the major ferroxidases involved in the oxidation of Fe(II) to Fe(III). On the other hand, free Fe(III) does not exist in sera, where it is almost completely found in a complex with transferrin (T) (*4*, *5*). When T is Fe(III)-loaded (holotransferrin, TFe_2), it interacts with transferrin receptor 1, which leads to the delivery of metal to the cell by receptor-mediated endocytosis (*6*). It has been suggested that a protein−protein interaction occurs between Cp and transferrin (T), which leads to the transfer of Fe(III) to T (*7*). This is supported by the fact that a multicopper ferroxidase (D-Fox) forms a stable adduct with transferrin in algae (*8*). Moreover, another member of the transferrin family, lactoferrin (Lf), is known to interact with Cp (*9*, *10*). Both proteins coexist in the colostrums and secretory granules of neutrophyles (*11*). Nonetheless, there is no evidence of an interaction of T or TFe_2 with Cp. In this work, we use emission spectroscopy to revisit the interaction between lactoferrin and ceruloplasmin and show for the first time the occurrence of a protein−protein adduct between holo- and/or apotransferrin and ceruloplasmin. These techniques already allowed us to deal with the thermodynamics of the interaction of metal-loaded transferrins (Fe, Al, Ga, Bi, Co, and U) with receptor 1 (*12*−*18*).

Human lactoferrin, holotransferrin, and apotransferrin were purified as previously described (*19*, *20*). Ceruloplasmin was provided by Euromedex and used without further purification. Protein−protein interactions were investigated by titration of Cp by P (P being Lf, T, or TFe_2). These titrations were performed in 100 μL of black-masking quartz microcuvettes, in 50 mM Hepes, 20 mM $NaHCO_3$, and 130 mM KCl buffer.

*To whom correspondence should be addressed. E-mail: thanh.haduong@univ-paris-diderot.fr (N.-T.H.-D.) or chahine@univ-paris-diderot.fr (J.-M.E.H.C.). Phone: +33157277239 or +33157277238. Fax: +33157277263.

If we assume that CpP is formed, we can write eqs 1 and 2 (*14*):

$$Cp + P \rightleftharpoons CpP \quad (1)$$

where $K_{obs} = [Cp][P]/[CpP]$.

$$c_1/[CpP] = 1 + K_{obs}/(c_2 - [CpP]) \quad (2)$$

where c_1 and c_2 are the analytical concentrations of Cp and P, respectively.

P and Cp possess typical fluorescence emission spectra (Figure S1 of the Supporting Information, P = Lf). In our case, because $c_1 \leq 1.7\ \mu M$ and $c_2 \leq 30\ \mu M$, at a fixed wavelength, the fluorescence emission of Cp (F_0) is proportional to c_1 ($F_0 = f_1[Cp]$), whereas that of P (F_P) would obey a quadratic equation ($F_P = B_1[P] + B_2[P]^2$) (*21*), where f_1, B_1, and B_2 are the experimental factors that associate, at a fixed wavelength, the fluorescence intensity with [Cp] and [P].

If CpP is formed according to eq 1, we can write eq 3 (*14*, *21*):

$$-B_2[CpP]^2 + (f_1 + B_1 + 2c_2B_2 - f_3)[CpP] + (\Delta F - f_1c_1 - B_1c_2 - B_2c_2^2) = 0 \quad (3)$$

where $\Delta F = F - F_0$ (F being the fluorescence intensity), f_3 is the experimental factor that relates the fluorescence to CpP, and [CpP] is the positive solution of quadratic eq 3.

At a fixed pH, adding iron-saturated lactoferrin to a solution of Cp leads to an increase in the fluorescence intensity with a 334 to 331 nm blue shift in the emission maximum, λ_{em} (Figure S1 of the Supporting Information). The titration curve of Cp by Lf ($0.2\ \mu M \leq c_1 \leq 0.4\ \mu M$) shows a slight increase in the fluorescence intensity at a λ_{em} of 334 nm that reaches a plateau when $c_2 \approx 4\ \mu M$ (Figure 1A), which is followed, when $c_2 \geq 6\ \mu M$, by a decrease in the emission intensity (Figure 1B). We shall, therefore, assume that two interactions occur between Lf and Cp. When $c_2 \leq 4\ \mu M$, a monoprotein−protein adduct (Lf−Cp) exists, whereas when $c_2 \geq 6\ \mu M$, a biadduct (2Lf−Cp) is formed. Both mono- and biadducts were reported in vitro, whereas only the monoadduct was found in vivo (*9*, *22*).

The experiment described in Figure 1A was repeated at five fixed pH values and a fixed ionic strength ($\mu = 0.2$). Five good linear least-squares regressions of the data against eq 2 were obtained (Figure 2A). From the slope of the best regression lines, five values of K_{obs} were determined. A plot of K_{obs} versus $1/[H^+]^n$ shows a linear relationship when $n = 1$ (Figure 2B). This implies the involvement of a single proton transfer in the process (*14*). We shall then assume that the protein−protein interaction occurs between the protonated form of Lf and Cp (eqs 4 and 5) (Supporting Information):

$$Lf + H^+ \rightleftharpoons (Lf)' \quad (4)$$

$$(Lf)' + Cp \rightleftharpoons CpLf \quad (5)$$

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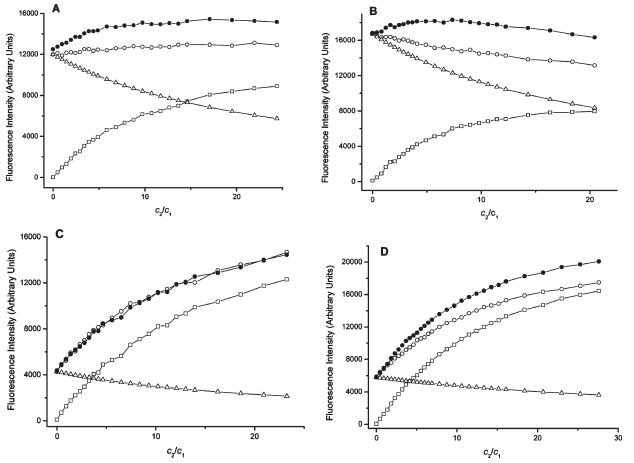

FIGURE 1: Variation of fluorescence emission of a solution of lactoferrin and/or apotransferrin in the presence and absence of ceruloplasmin, with c_2/c_1 after subtraction of the emission contribution of the buffer: (□) c_2, (△) c_1, (○) c_1 in the presence of c_2, and (●) theoretical sum of the fluorescence emission contributions of c_1 and c_2 at a λ_{ex} of 280 nm and a λ_{em} of 334 nm (μ = 0.2 at 37 ± 0.1 °C). (A) For lactoferrin, 0.2 μM < c_1 < 0.4 μM and 0.19 μM < c_2 < 4.88 μM at pH 7.4. (B) For lactoferrin, 0.8 μM < c_1 < 1.6 μM and 0.64 μM < c_2 < 16.35 μM at pH 7.4. (C) For apotransferrin, 0.2 μM < c_1 < 0.4 μM and 0.18 μM < c_2 < 4.65 μM at pH 7.6. (D) For apotransferrin, 0.8 μM < c_1 < 1.6 μM and 0.168 μM < c_2 < 25.8 μM at pH 7.1.

where (Lf)′ is the protonated form of lactoferrin, $K_a = [Lf][H^+]/[(Lf)']$, and $K_{11Lf} = [(Lf)'][Cp]/[CpLf]$. In this case, K_{obs} can be expressed as shown in eq 6 (Supporting Information).

$$K_{obs} = K_{11Lf} + K_a K_{11Lf}/[H^+] \quad (6)$$

From the intercept and the slope of the best line of Figure 2B, $K_{11Lf} = (1.5 \pm 0.2) \times 10^{-6}$ M and $K_a = (6.3 \pm 1.6) \times 10^{-8}$ M. The K_a value is, within the limits of uncertainty, identical to that determined by acid−base spectrophotometric titration (Supporting Information). As for K_{11Lf}, it is within the limits of uncertainty identical to that already reported for CpLf formation in vivo as well as in vitro (*9*, *10*, *22*). CpLf is ionic and dissociates at pH ≤4.7, or at a high salt concentration (*22*). We confirmed this fact by performing a series of experiments at a different ionic strength (μ = 0.5). At pH 6.9, K_{obs} increases from 1.77×10^{-6} M at μ = 0.2 to 7.76×10^{-6} M at μ = 0.5. This can related to the fact that lactoferrin is positively charged (pI 8.7) (*23*) whereas ceruloplasmin is negatively charged (pI 4.4) (*24*).

When 0.8 μM ≤ c_1 ≤ 1.6 μM and c_2 ≥ 6 μM, the emission intensity of the titration curve starts to decrease (Figure 1B). We assumed that this describes the formation of the biadduct (2Lf−Cp), recently detected by gel filtration (*10*). The dissociation constant has never been reported. We determine it here from the titration curves of Figure 1B. The K'_{obs} values were identical at the five pH values: $K_{12Lf} = [CpLf][Lf]/[CpLf_2] = (1.1 \pm 0.2) \times 10^{-5}$ M. Lf, therefore, possesses two Cp binding sites, the second of which is independent of pH and ionic strength.

Revisiting lactoferrin was necessary to validate our approach for investigating the interaction between ceruloplasmin and human serum transferrin. The same experiments were, therefore, performed for the interaction of Cp with iron-saturated transferrin (holotransferrin, TFe_2) or apotransferrin (T). No interaction between TFe_2 or T and Cp is observed in the concentration range used with Lf (0.2 μM < c_1 < 0.4 μM, and 0.18 μM < c_2 < 4.65 μM) (Figure 1C). However, adding TFe_2 or T at higher concentrations (0.2 μM ≤ c_2 ≤ 26 μM) to a solution of Cp leads

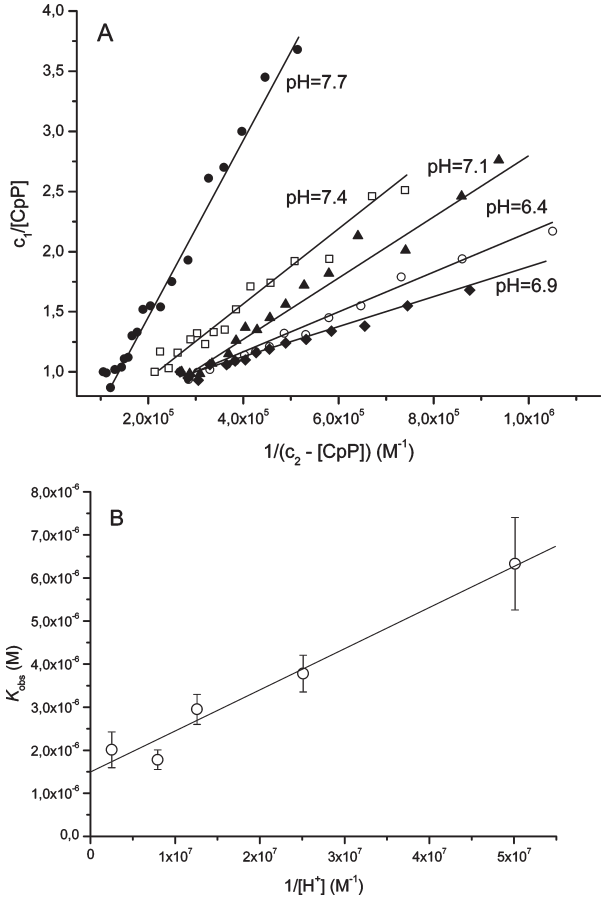

FIGURE 2: Dissociation constant of the 1:1 Lf−Cp protein−protein adduct. (A) Plot of $c_1/[CpP]$ vs $1/(c_2 - [CpP])$ where 0.2 μM < c_1 < 0.4 μM, 0.19 μM < c_2 < 4.88 μM, and μ = 0.2 at 37 ± 0.1 °C at five fixed pH values. (B) Plot of K_{obs} vs $1/[H^+]$ at μ = 0.2. The intercept is (1.5 ± 0.2) × 10^{-6} M. The slope is (9.5 ± 0.9) × 10^{-14} M^2, and r = 0.9858.

to a decrease or increase in the fluorescence emission, respectively (Figure S3 of the Supporting Information and Figure 1D), as observed for the interactions of some metal-loaded transferrin with receptor 1 (*12*−*18*). As in the case of Lf, this implies the formation of a protein−protein adduct between Cp and TFe_2 or T. A good linear regression of the data against eq 2 was obtained. From the slopes of the best regression lines, identical K_{obs} values were obtained at different pH values, implying that the process is pH-independent: $K_{1T} = (1.9 \pm 0.7) \times 10^{-5}$ M, and $K_{1TFe2} = (1.2 \pm 0.4) \times 10^{-5}$ M. If as in the case of Lf, an interaction is to occur between T and CpT, the associated dissociation constant $K_{2T} \gg K_{1T}$. This would require working at a c_1 of ∼0.1 mM, which is not compatible with fluorescence detection. The K_{1P} (P = T or TFe_2) values are ∼10-fold higher than those reported for the monoadduct (Lf−Cp) but are in the range of dissociation constants found for the ferritin and ceruloplasmin complex (*25*). For further confirmation, differential absorption spectra were recorded for Cp in the presence of a very large excess of T or TFe_2 (10-fold) in both reference and sample cells. This allowed the determination of the absorption spectra of both $CpTFe_2$ and CpT (Supporting Information). These differ from those of T and Cp [blue shift from 278 to 275 nm (Figure 3)]. The high values of K_{1T} and K_{1TFe2} explain why Hudson et al. (*7*) were not able to detect the ceruloplasmin−transferrin complex by native polyacrylamide gel electrophoresis.

We have shown here that two interactions exist between Cp and T or TFe_2. These interactions are weaker than with Lf,

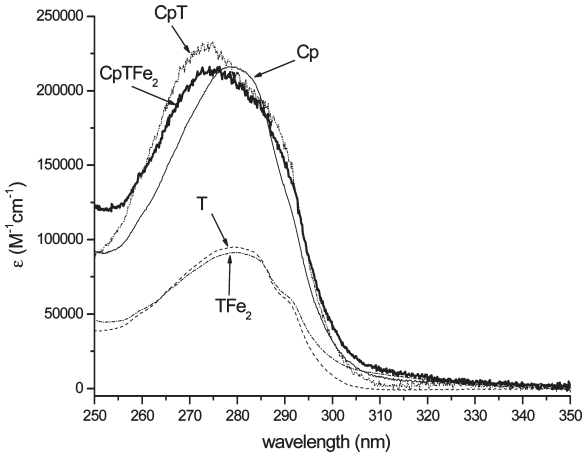

FIGURE 3: Absorption coefficient spectra of apotransferrin (T), holotransferrin (TFe_2), ceruloplasmin (Cp), and the ceruloplasmin−apotransferrin (CpT) and ceruloplasmin−holotransferrin ($CpTFe_2$) adducts. The spectra were recorded at 37 °C, μ = 0.2, and pH 7.4 (Supporting Information).

probably because in neutral media the overall charges of Lf and Cp are opposite, whereas they are both negative with T and Cp (*4*, *24*). Nevertheless, in the bloodstream, Cp and T concentrations (2.5 and 25 μM, respectively) (*26*, *27*) are sufficiently high for the formation of the protein−protein adducts. Therefore, a cautious transposition of our in vitro results to serum indicates that 70% of the Cp would be in interaction with transferrin. Whether this interaction confirms the transfer of Fe(III) from Cp to T is far from proven. Nevertheless, the fact that this interaction is weak leads to the prevention of any competition with that occurring between TFe_2 and receptor 1 (*14*). Therefore, the presence of $CpTFe_2$ in the medium will not interfere with the acquisition of iron by the receptor-mediated endocytosis pathway.

SUPPORTING INFORMATION AVAILABLE

Supporting results, experimental procedures, and Figures S1−S3. This material is available free of charge via the Internet at http://pubs.acs.org.

REFERENCES

1. Arredondo, M., and Nunez, M. T. (2005) *Mol. Aspects Med. 26*, 313–327.
2. Hellman, N. E., and Gitlin, J. D. (2002) *Annu. Rev. Nutr. 22*, 439–458.
3. Lindley, P. F., Card, G., Zaitseva, I., Zaitsev, V., Reinhammar, B., Selin-Lindgren, E., and Yoshida, K. (1997) *J. Biol. Inorg. Chem. 2*, 454–463.
4. Sun, H., Li, H., and Sadler, P. J. (1999) *Chem. Rev. 99*, 2817–2842.
5. Crichton, R. R. (2001) Inorganic biochemistry of iron metabolism, John Wiley & Sons, West Sussex, U.K.
6. Dautry-Varsat, A., Ciechanover, A., and Lodish, H. F. (1983) *Proc. Natl. Acad. Sci. U.S.A. 80*, 2258–2262.
7. Hudson, D. M., Krisinger, M. J., Griffiths, T. A., and Macgillivray, R. T. (2008) *J. Cell. Biochem. 103*, 1849–1855.
8. Paz, Y., Katz, A., and Pick, U. (2007) *J. Biol. Chem. 282*, 8658–8666.
9. Sabatucci, A., Vachette, P., Vasilyev, V. B., Beltramini, M., Sokolov, A., Pulina, M., Salvato, B., Angelucci, C. B., Maccarrone, M., Cozzani, I., and Dainese, E. (2007) *J. Mol. Biol. 371*, 1038–1046.
10. Zakharova, E. T., Shavlovski, M. M., Bass, M. G., Gridasova, A. A., Pulina, M. O., De Filippis, V., Beltramini, M., Di Muro, P., Salvato, B., Fontana, A., Vasilyev, V. B., and Gaitskhoki, V. S. (2000) *Arch. Biochem. Biophys. 374*, 222–228.
11. Sokolov, A. V., Ageeva, K. V., Pulina, M. O., Zakharova, E. T., and Vasilyev, V. B. (2009) *BioMetals 22*, 521–529.
12. Chikh, Z., Ha-Duong, N. T., Miquel, G., and El Hage Chahine, J. M. (2007) *J. Biol. Inorg. Chem. 12*, 90–100.
13. Chikh, Z., Hémadi, M., Miquel, G., Ha-Duong, N. T., and El Hage Chahine, J. M. (2008) *J. Mol. Biol. 380*, 900–916.
14. Hémadi, M., Kahn, P. H., Miquel, G., and El Hage Chahine, J. M. (2004) *Biochemistry 43*, 1736–1745.
15. Hémadi, M., Miquel, G., Kahn, P. H., and El Hage Chahine, J. M. (2003) *Biochemistry 42*, 3120–3130.
16. Miquel, G., Nekaa, T., Kahn, P. H., Hémadi, M., and El Hage Chahine, J. M. (2004) *Biochemistry 43*, 14722–14731.
17. Ha-Duong, N. T., Hémadi, M., Chikh, Z., and Chahine, J. M. (2008) *Biochem. Soc. Trans. 36*, 1422–1426.
18. Hémadi, M., Ha-Duong, N. T., Plantevin, S., Vidaud, C., and El Hage Chahine, J. M. (2010) *J. Biol. Inorg. Chem. 15*, 497–504.
19. Pakdaman, R., and El Hage Chahine, J. M. (1997) *Eur. J. Biochem. 249*, 149–155.
20. Pakdaman, R., Abdallah, F. B., and El Hage Chahine, J. M. (1999) *J. Mol. Biol. 293*, 1273–1284.
21. Valeur, B. (2001) Molecular fluorescence: Principles and applications, Wiley-VCH, New York.
22. Pulina, M. O., Zakharova, E. T., Sokolov, A. V., Shavlovski, M. M., Bass, M. G., Solovyov, K. V., Kokryakov, V. N., and Vasilyev, V. B. (2002) *Biochem. Cell Biol. 80*, 35–39.
23. Moguilevsky, N., Retegui, L. A., and Masson, P. L. (1985) *Biochem. J. 229*, 353–359.
24. Oosthuizen, M. M., Nel, L., Myburgh, J. A., and Crookes, R. L. (1985) *Anal. Biochem. 146*, 1–6.
25. Reilly, C. A., Sorlie, M., and Aust, S. D. (1998) *Arch. Biochem. Biophys. 354*, 165–171.
26. Weinberg, E. D. (1978) *Microbiol. Rev. 42*, 45–66.
27. Morell, A. G., Van den Hamer, C. J., and Scheinberg, I. H. (1969) *J. Biol. Chem. 244*, 3494–3496.