Thiophilic Interaction Chromatography of Mammalian and Avian Transferrins

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Abstract

Transferrins are a class of iron-binding proteins widely distributed in biological fluids. All transferrins possess two metal binding sites, each of which can bind a ferric iron. Transferrins play a major role in plasma iron transport and have anti-bacterial, anti-inflammatory, and immunological functions. Lactoferrin is an iron-binding bilobal protein of the transferrin family found in neutrophilic leukocytes and external secretion of mammals. In an earlier communication, we have shown that both human serum transferrin and lactoferrin bind to 3S-gel. Ovotransferrin, the major egg-white protein, is an avian transferrin. In this paper, the details of the binding of mammalian and avian transferrins to 2S gel is presented. Both, *apo* and *holo* ovotransferrin, bind to 2S T-gel. *Holo* and *apo* lactoferrin from other mammalian species such as cow, rabbit, dog, mouse, and rat bind to T-gel.

Introduction

Transferrins are a class of iron-binding proteins widely distributed in biological fluids (1,2). Serum transferrin is the principle iron-binding protein in vertebrates (3). It is a bilobal 80 kDa protein and has two binding sites for iron, one each in the N and C lobes. The two iron-binding sites are structurally similar, and bind iron strongly at physiologic pH in a process that involves a great deal of tertiary structural rearrangement (4). The rate of release of iron from serum transferrin depends on factors such as pH, concentration and type of ionic species present, and temperature. It transports iron from sites of absorption and heme degradation to sites for storage and utilization. It plays a role in the stimulation of cell growth (5) and is capable of binding iron and maintaining it in a relatively non-reactive state (6) thereby providing an anti-oxidant defense.

Lactoferrin is an iron-binding protein found in blood, milk, and exocrine secretions including nasal, pancreatic juice, lachrymal, vaginal, seminal, and specific granules of neutrophils (7). It is highly basic and interacts with many acidic molecules (8). Its molecular weight is 80 kDa and is composed of 703 amino acid residues (9). It is a potent inhibitor of colony stimulating factor (CSF) and has a nutritional, bacteriostatic, and immuno-

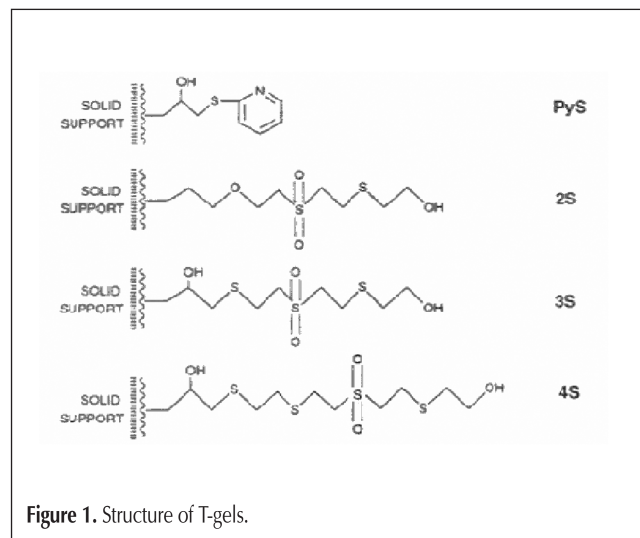

Figure 1. Structure of T-gels.

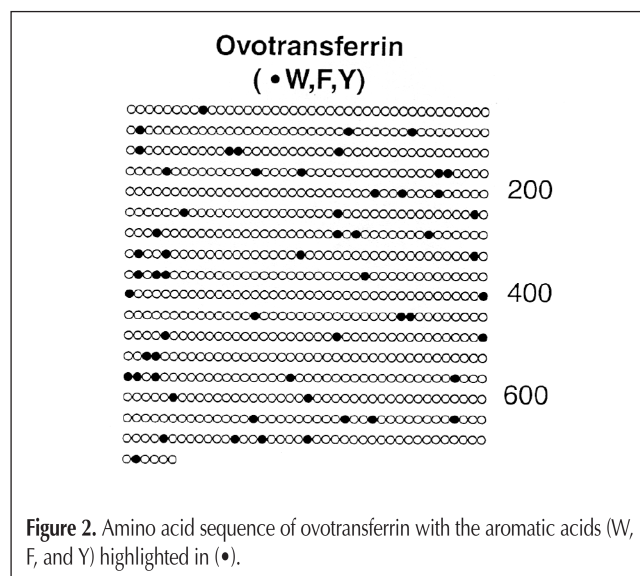

Figure 2. Amino acid sequence of ovotransferrin with the aromatic acids (W, F, and Y) highlighted in (•).

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logical role in the body. Lactoferrin's main function is to provide a primary defense against microbial infection, mainly due to its ability to sequester iron that is necessary for microbial growth. In vitro, lactoferrin has been shown to inhibit several strains of bacteria, including *Staphylococcus aureus* and *Escherichia. coli*. This anti-bacterial effect is lost when lactoferrin is saturated with iron. Lactoferrin can be given as a nutritional supplement to any adult and can be given specifically to people with iron deficiencies. Lactoferrin may be involved in the development of a variety of neurodegenerative disorders; it has been found in senile plaques and neurofibrillary tangles of Alzheimer's disease (10) and in some microglia, astrocytes, and oligodendrocytes (11). It is found in breast milk, in epithelial secretions, and also in the secondary granules of neutrophils (12). Lactoferrin is a pleiotropic factor with potent antimicrobial and immuno-modulatory activities, and recent studies have demonstrated that it promotes bone growth (13).

Ovotransferrin, the major egg-white protein, is a member of the transferrin family of iron-binding proteins (14,15). Ovotransferrin comprises of 686 amino-acid residues, is folded into two homologous lobes (N and C terminal lobes) with two distinct domains; two metal-binding sites are located within the inter-domain clefts of each lobe (16,17).

Thiophilic interaction chromatography (TIC) was introduced by Jerker Porath and coworkers in 1985 (18). It has also been shown that TIC is an effective method for the isolation of the proteins containing surface localized clusters of aromatic residues, such as tryptophan, phenylalanine and tyrosine (19–20). We have been evaluating the binding avidities of several proteins to thiophilic gels and their use for the purification of some cancer biomarkers. Quite recently, TIC has been applied to purify several proteins, including the prostate specific antigen (PSA) at Roswell Park Cancer Institute (21–23). In an earlier communication from our laboratory, we reported the TIC of human serum transferrin and lactoferrin and its potential application to quantify the levels of these transferrins in serum of Alzheimer's patients (24). TIC of amyloid β-peptides of interest in Alzheimer's disease has also been reported from our laboratory (25). Although the mammalian and avian transferrins bind to the thiophilic gels (PyS, 2S and 3S), we report only the details of the binding to 2S gel in this paper.

Experimental

The following components constituted the high-performance liquid chromatography (HPLC) setup used for TIC: Waters 600 multi-solvent delivery system from Millipore Corporation (Waters Millipore, Milford, MA) with 200 µL pumpheads; rheodyne Model 7125 syringe loading sample injector with a 2 mL sample loop; Waters 994 programmable photodiode array detector; Waters 470 fluorescence detector; microflow cell and pH microelectrode from Amersham Pharmacia Biotechnolgy (Piscataway, NJ); baseline 810 chromatography work station software from Millipore Corporation installed on a HP vectra PC (Hewlett Packard, Palo Alto, CA).

The proteins were detected using the Waters 994 photodiode detector. The detector monitored absorbances from 200 nm to 400 nm. The plot of the spectra was acquired using the software from Waters 470 fluorescence detector. The solvents used for the experiments were (1M Na_2SO_4, 20mM PO_4 at pH 7.4) and (20mM phosphate buffer, pH 7.4). The column was washed with 10 column volumes (30 mL) of starting solution (1M Na_2SO_4, 20 mM PO_4 at pH 7.4) before each experiment. The transferrins bind to a T-gel at high concentration of lyotropic salt and are released as its level was decreased. All TIC of transferrins was done with a linear gradient: 0–10 min (1M Na_2SO_4, 20mM PO_4, pH 7.4) and 10–60 min. A linear gradient from starting solution to 20mM phosphate buffer alone was applied at 60 min.

The different samples of mammalian lactoferrin and serum transferrin were purchased from Sigma Aldrich Chemicals (St. Louis, MO). T-gel columns were purchased from Amersham Pharmacia Biotechnolgy. The PyS and 2S gels were purchased from Sigma Aldrich Chemicals and the 3S gel was obtained from EM Scientific Company (Carson City, NV). All samples of trans-

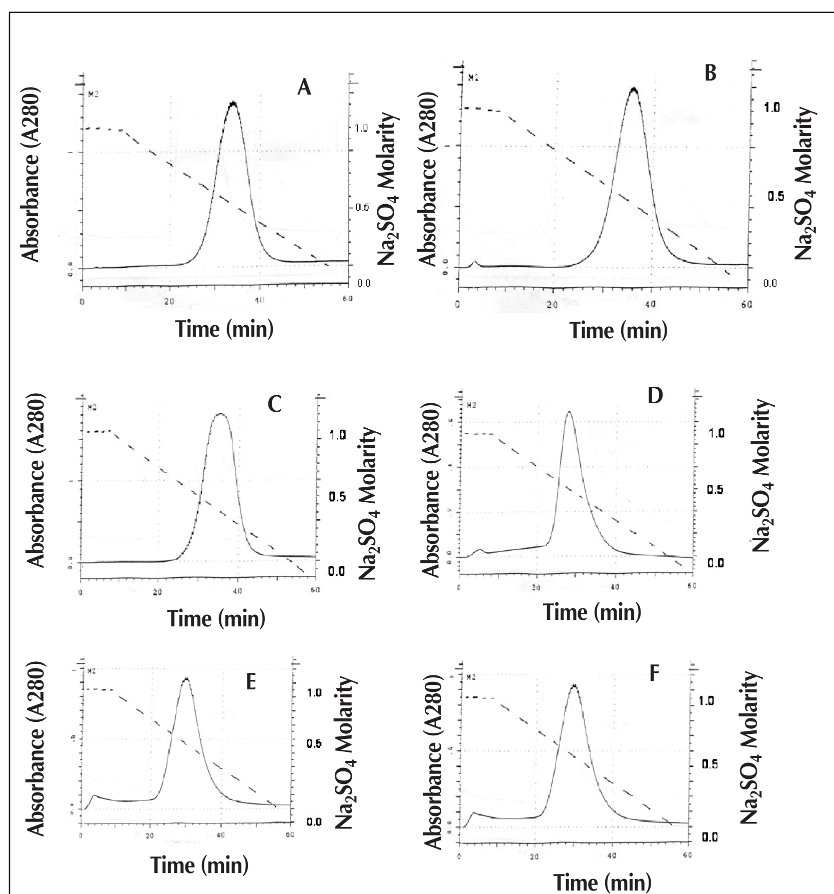

Figure 4. TIC of serum transferrin (A), lactoferrin (B), and ovotransferrin (C) (Apo and holo forms) on 2S run at a flow rate of 0.5mL/min (0–10 min in 1M sodium sulfate with 20mM phosphate buffer at pH 7.4 and linear gradient from 10–60 min).

ferrins were prepared by dissolving 4 mg of commercially purchased protein in 1 mL of 1M Na_2SO_4, 20mM PO_4 at pH 7.4. Prior to the injection of the sample, the sample was centrifuged. An HR 10/2 column was purchased from Amersham Pharmacia Biotechnolgy. The column was designed for use on a fast performance liquid chromatography (FPLC) system and appropriate FPLC/HPLC unions (P 626 10-32 adapter) were purchased from Upchurch Scientific, Inc (Oak Harbor, WA). This column was packed with PyS, 2S, and 3S thiophilic gels. All experiments were done at a constant flow rate of 0.5 mL/min at room temperature (20°C). All solvents were degassed and purged using helium gas in accordance with the Waters recommendations. Although all transferrins bind to all three T-gels, we present and discuss the binding of these proteins to only one of the gels, the 2S gel.

Results and Discussion

Figure 1 gives the structures of four thiophilic gels, PyS, 2S, 3S, and 4S gels (20). Figure 2 presents the distribution of the aromatic residues (W, F, and Y) in ovotransferrin as solid dots (•). Figures 3A and 3B (See page 5A) show a view of the tertiary structures of aluminum bound ovotransferrin (16) and hen *apo*-ovotransferrin (17), showing the presence of several exposed aromatic residues on their surface. All non-aromatic amino acids are shown in blue and aromatic amino acids Y in yellow, F in magenta, and W in red. Thus, ovotransferrin satisfies the binding requirement to thiophilic gels (19,20). Figures 4A, 4B, and 4C give the TIC of human serum transferrin, human lactoferrin, and ovotransferrin (*apo* and *holo* forms) run on 2S gel. Figure 5 presents the binding of *apo*-transferrins from several mammalian sources such as cow, rat, rabbit, dog, and mouse (26).

Conclusions

The affinity of human serum transferrin and lactoferrin for 3S gel has been previously reported (24). That observation is now extended to several mammalian transferrins and an avian ovotransferrin. Moreover, we now report that all transferrins display a binding affinity for T-gel. The binding to these gels occurs with *holo* and *apo* transferrins with similar affinity as revealed by the linear gradient elution. The identity of the binding sites, presumably containing aromatic clusters, remains to be established. The use of TIC in quantitation of human serum transferrin in Alzheimer's patients is currently in progress in our laboratory and will be published in the near future.

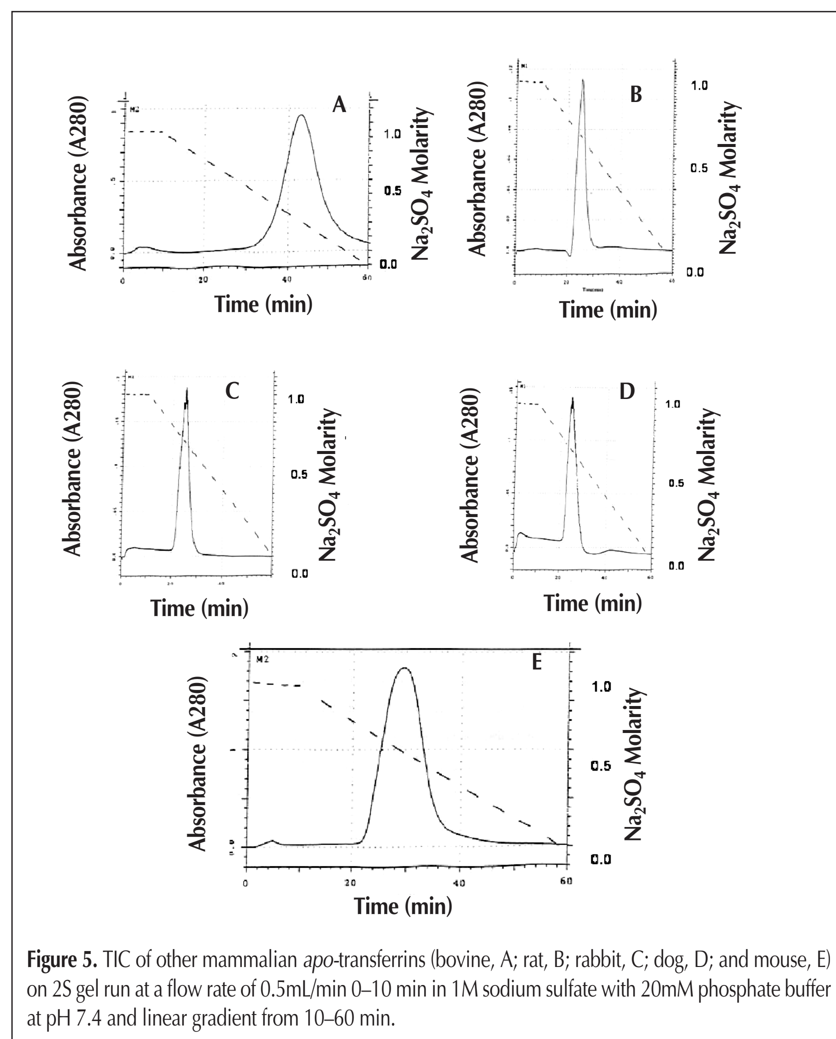

Figure 5. TIC of other mammalian *apo*-transferrins (bovine, A; rat, B; rabbit, C; dog, D; and mouse, E) on 2S gel run at a flow rate of 0.5mL/min 0–10 min in 1M sodium sulfate with 20mM phosphate buffer at pH 7.4 and linear gradient from 10–60 min.

References

1. L.A. Lambert, H. Perri, and T.J. Meehan. Evolution of duplications in the transferrin family of proteins. *Comp. Biochem. Physiol., B* **140:** 11–25 (2005).
2. L.A. Lambert, H. Perri, P.J. Halbrooks, and A.B. Mason. Evolution of the transferrin family: conservation of residues associated with iron and anion binding. *Comp. Biochem. Physiol., B* **142:** 129–141 (2005).
3. M. Belew, N. Juntti, A. Larsson, and J. Porath. A one-step purification method for monoclonal antibodies based on salt-promoted adsorption chromatography on a 'thiophilic' adsorbent. *J. Immunol. Methods* **102:** 173–182 (1987).
4. S.L. Mecklenburg, R.J. Donohoe, and G.A. Olah. Tertiary structural changes and iron release from human serum transferrin. *J. Mol. Biol.* **270:** 739–750 (1997).
5. S. Bailey, R.W. Evans, R.C. Garratt, B. Gorinsky, S. Hasnain, C. Horsburgh, H. Jhoti, P.F. Lindley, A. Mydin, and R. Sarra. Molecular structure of serum transferrin at 3.3-Å resolution. *Biochemistry* **27:** 5804–5812 (1988).
6. J. Thome, W. Gsell, M. Rosler, J. Kornhuber, L. Frolich, E. Hashimoto, B. Zielke, G.A. Wiesbeck, and P. Riederer. Oxidative-stress associated parameters (lactoferrin, superoxide dismutases) in serum of patients with Alzheimer's disease. *Life Sciences* **60:** 13–19 (1997).
7. E.N. Baker, B.F. Anderson, H.M. Baker, M. Haridas, G.B. Jameson, G.E. Norris, S.V. Rumball, and C.A. Smith. Structure, function and flexibility of human lactoferrin. *Int. J. Macromol.* **13:** 122–129 (1991).
8. A. Rosenau, F. Chelu, M. Trif, C. Motas, and J.H. Brock. Inhibition of binding of lactoferrin to

the human promonocyte cell line THP-1 by heparin: the role of cell surface sulphated molecules. *Biochim. Biophys. Acta.* **1475:** 35–38 (2000).

9. M.H. Metz-Boutigue, J. Jolles, J. Mazurier, F. Schoentgen, D. Legrand, G. Spik, J. Montreuil, and P. Jolles, P. Human lactotransferrin: amino acid sequence and structural comparisons with other transferrins. *Eur. J. Biochem.* **145:** 659–676 (1984).
10. P. Fischer, M.E. Gotz, W. Danielczyk, W. Gsell, and P. Riederer. Blood transferrin and ferritin in Alzheimer's disease. *Life Sciences* **60:** 2273–2278 (1997).
11. T. Kawamata, I. Tooyama, T. Yamada, D.G. Walker, and P.L. McGeer. Lactotransferrin immunocytochemistry in Alzheimer and normal human brain. *Amer. J. Pathol.* **142:** 1574–1585 (1993).
12. B. Lonnerdal and S. Iyer. Lactoferrin: molecular structure and biological function. *Annu. Rev. Nutr.* **15:** 93–110 (1995).
13. D. Naot, A. Grey, I.R. Reid, and J. Cornish. Lactoferrin—a novel bone growth factor. *Clin. Med. Res.* **3:** 93–101 (2005).
14. P. Aisen and I. Listowsky. Iron transport and storage proteins. *Annu. Rev. Biochem.* **49:** 357–393 (1980).
15. H.M. Baker, B.F. Anderson, and E.N. Baker. Dealing with iron: common structural principles in proteins that transport iron and heme. *Proc. Natl. Acad. Sci. USA* **100:** 3579–3583 (2003).
16. K. Mizutani, B. Mikami, S. Aibara, and M. Hirose. Structure of aluminium-bound ovotransferrin at 2.15 Angstroms resolution. *Acta Crystallogr.* **D61:** 1636–1642 (2005).
17. P.G. Thakurta, D. Choudhury, R. Dasgupta, and J.K. Dattagupta. Tertiary structural changes associated with iron binding and release in hen serum transferrin: a crystallographic and spectroscopic study. *Biochem. Biophys. Comm.* **316:** 1124–1131 (2004).
18. J. Porath, F. Maisano and M. Belew. Thiophilic adsorption—a new method for protein fractionation. *FEBS Lett.* **185:** 306–310 (1985).
19. T. W. Hutchens and J. Porath. Thiophilic adsorption: a comparison of model protein behavior. *Biochemistry* **26:** 7199–7204 (1987).
20. A. Schwarz and M. Wilchek. Possible sites on antibodies involved in thiophilic adsorption. *Advances in Molecular & Cellular Biology* **15B:** 547–551 (1996).
21. K.C. Chadha, E. Kawinski, and E. Sulkowski. Thiophilic interaction chromatography of prostate-specific antigen. *J. Chromatogr. B* **754:** 521–525 (2001).
22. B. Kumar, E. Kawinski, C. Cherrin, L. M. Gambino, M. P. Nair, S. A. Schwartz, and K. C. Chadha. Two step procedure for purification of enzymatically active prostate- specific antigen from seminal plasma. *J. Chromatogr. B* **710:** 57–65 (2004).
23. B. Kumar, S.A. Schwartz, M.P. Nair, R. Aalinkeel, E. Kawinski, and K.C. Chadha. Prostate-specific antigen modulates the expression of genes involved in prostae tumor growth. *Neoplasia* **7:** 241–252 (2004).
24. T. Srikrishnan, J.T. MacKenzie, and E. Sulkowski. Thiophilic interaction chromatography of human transferrins. *J. Chromatogr. Sci.* **44:** 634–638 (2006).
25. S. Parry, D. Todorova-Balvay, T. Srikrishnan, and E. Sulkowski. Thiophilic interaction chromatography of Alzheimer's beta-amyloid peptides. *J. Pep. Res., Suppl.* **1:** 99–105 (2006).
26. J.T. MacKenzie. Thiophilic Interaction Chromatography of Transferrins, thesis submitted to State University of New York at Buffalo, 2000.

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