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Short Communication

High-performance liquid chromatographic separation of human apotransferrin and monoferric and diferric transferrins

YOSHIAKI MAKINO* and ETUKO KAWANISHI

Department of Biochemistry, School of Medicine, Showa University, 1-5-8 Hatanodai, Shinagawa-ku, Tokyo 142 (Japan)

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ABSTRACT

A simple method for the chromatographic separation of the different molecular forms of human transferrin according to their respective iron contents is described. The appropriate conditions were developed with a Mono-S cation-exchange column.

INTRODUCTION

Transferrin, a protein found in the blood of all vertebrate species, has a relative molecular mass in the range of 75 000–80 000 depending on the species, but appears to be composed of a single polypeptide chain in all cases. Transferrin has two iron binding sites. As iron is bound to these sites, bicarbonate is bound to an anion-binding site close to each of the iron binding sites [1,2]. These sites, however, are not equivalent with respect to iron binding: site A, which is located at the C-terminal side of transferrin (domain CD2) has a higher binding affinity for iron than site B, which is located at the N-terminal side of the protein (domain ND2).

Transferrin that contains iron (not iron-saturated) has been separated into two components by electrophoresis [3] and three compounds by isoelectric focusing [4–6]. Makey and Seal [7] and Leibman and Aisen [8] reported the separation of four molecular forms of transferrin by electrophoresis in polyacrylamide gel containing 6 *M* urea. These separations appear to be based directly on the amount of iron bound by transferrin, *i.e.*, iron-free, A-site iron-bound, B-site iron-bound or iron-saturated [9]. Iron influences the isoelectric point of the transferrin. By isoelectric focusing, diferric transferrin from human serum can be separated into different isoelectric point forms. Those forms have different carbohydrate parts,

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especially different amounts of N-acetylneuraminic acid, also denoted sialic acid [10–13].

The aim of the present study was to develop a new direct method for the separation of the different molecular forms of transferrin using high-performance liquid chromatography (HPLC).

EXPERIMENTAL

Materials

The materials were purchased from the following suppliers: Mono-S HR 5/5 ion-exchange HPLC column from Pharmacia Fine Chemicals (Uppsala, Sweden); TSK G 2000 gel filtration column from LKB-Productor (Bromma, Sweden); acrylamide and bisacrylamide from Wako (Osaka, Japan); urea from Nakalai Tesque (Kyoto, Japan). Water for HPLC analysis was twice distilled in glass.

Purification of human transferrin

Human transferrin was purified from pooled plasma using preparative polyacrylamide gel electrophoresis [6] and gel permeation on a TSK G 2000 HPLC chromatographic column.

Preparation of apotransferrin

Human transferrin, isolated from the above method, was used to prepare apotransferrin by sequential dialysis of 50 mg of transferrin disolved in 5 ml of water against 200 ml of each of the following solutions at 4°C: 0.1 *M* sodium citrate–0.1 *M* sodium acetate, pH 4.5 (four times); deionized water (twice); 0.1 *M* $NaClO_4$ (three times); deionized water (four times) [14].

Preparation of diferric and monoferric transferrin

Transferrin, purified by the above method, was dissolved in a small volume of 0.02 *M* sodium citrate buffer (pH 5.1). After 1 h, the solution was dialysed against 0.02 *M* $NaHCO_3$ for 24 h. After dialysis, $NaHCO_3$ was added to the transferrin solution to a final concentration of 0.6 *M*. A solution of 0.25% $FeCl_3$ in 0.1–0.5 *M* HCl was added to a twenty-fold excess in volume of 0.02 *M* sodium citrate buffer, and then slowly mixed with the transferrin solution. The high concentration of $NaHCO_3$ neutralized the added HCl, and the final pH was always between 7 and 8 [15]. As shown by Bates *et al.* [16], the iron–transferrin complex forms very rapidly in a 1:20 (v/v) iron–citrate buffer solution in the presence of excess bicarbonate. Unbound iron was removed after 1 h by dialysis against two changes of 0.02 *M* $NaHCO_3$.

Transferrin with various degrees of iron-saturation was obtained by the addition of an appropriate amount of $FeCl_3$ to apotransferrin in 0.02 *M* $NaHCO_3$.

Electrophoresis in 6 M urea

Electrophoresis in 6 *M* urea–polyacrylamide gels was carried out by methods previously described [8], using a vertical slab gel apparatus, with slabs measuring 2 × 110 × 135 mm. Gels of 2 mm thick in a vertical system were cast in 0.089 *M* Tris–0.089 *M* borate buffer (pH 8.4) containing 6 *M* urea and 50 m*M* EDTA. Pre-electrophoresis of 6 *M* urea–polyacrylamide gel was run at 75 V/cm for 30 min before the sample was applied. Samples containing from 30 to 60 μg of transferrins were applied to each slot of vertical gel slab. The sample buffer was prepared from 7.5 ml of 8 *M* urea, 0.5 ml of 1.8 *M* Tris–borate at pH 8.4 and 2 g of sucrose. To 0.87 ml of this buffer was added 0.13 ml of the sample to be analysed. A 30-μl sample of this mixture was applied to a slot of the urea–polyacrylamide gel slab. A gradient of 7.5 V/cm was employed for electrophoresis at 4°C for 16–17 h.

Ion-exchange column chromatography

A Mono-S HR 5/5 column was used in the LKB HPLC system (Urtrochrom GTi) consisting of a 2154 HPLC injector, a 2152 LC controller, a 2150 HPLC pump, a 2212 helirac, a 2210 recorder and a 2151 variable-wavelength monitor set at 280 nm. The inlet side of the column was fitted with additional tubing to act as a pre-column cooling coil. Then the column and cooling coil were immersed in a bath at 10°C. The column was equilibrated at 0.5 ml/min with 0.05 *M* sodium acetate buffer (pH 5.2), which was degassed and filtered through a 0.22-μm membrane filter (type Sterivex-GV, Millipore). The sample, which was dialysed against the above buffer in volumes up to 2 ml, was then loaded through the injection valve into a Superloop and introduced into the column. After sample application, the column was washed with the above buffer for 10 min. Transferrin adsorbed on the column was eluted stepwise with 40, 90 and 200 m*M* sodium chloride in 0.02 *M* acetate buffer (pH 5.4). At the end of the stepwise elution, the column was washed for 10 min with 1.0 *M* sodium chloride in the above buffer prior to returning to the initial conditions for the next sample.

RESULTS AND DISCUSSION

Transferrin was purified from human pooled plasma by procedures that have been described in detail elsewhere [6]. The purity of the purified transferrin was checked by sodium dodecylsulphate polyacrylamide gel electrophoresis (SDS-PAGE) (Fig. 1A) and immunoelectrophoresis (not shown), and it was then used as the starting material for all subsequent purification procedures (not shown). The HPLC profiles for the pure transferrin with different iron binding are shown in Fig. 2. The sample used for Fig. 2A contained apotransferrin, monoferric transferrin and diferric transferrin. Three peaks from the Mono-S column were eluted with 40, 90 and 200 m*M* sodium chloride in acetate buffer (pH 5.4). The samples used for Fig. 2B and C correspond respectively to diferric transferrin and

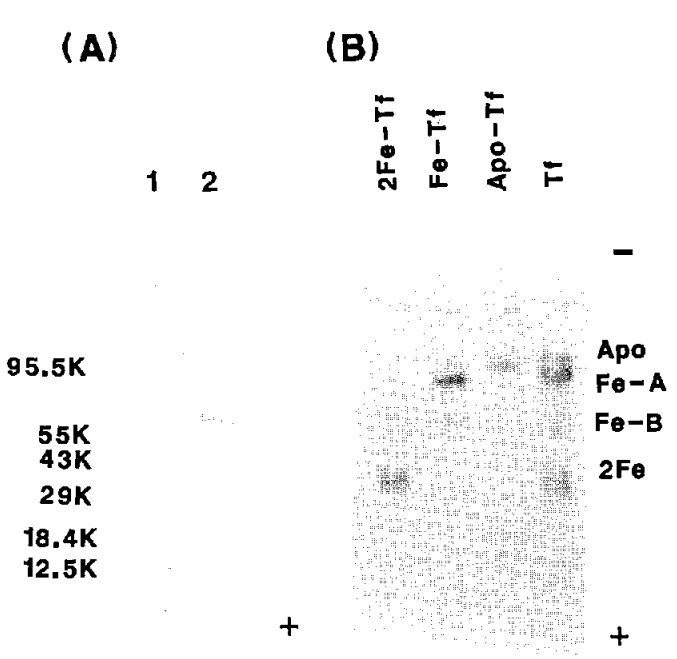

Fig. 1. (A) SDS-PAGE of purified transferrin. Lane 1, molecular mass markers; lane 2, purified transferrin. (B) Electrophoresis of human transferrin in a 6 *M* urea Tris borate–EDTA buffer (pH 8.4) system: Tf, approximately 60% iron saturation transferrin (unfractionated Tf); 2Fe-Tf, after HPLC diferric transferrin; Fe-Tf, after HPLC monoferric transferrin (A-site, B-site transferrin); Apo-Tf, after HPLC apotransferrin.

apotransferrin, prepared as described in Experimental. In both cases, a single transferrin peak was detected when the sodium chloride concentration reached 40 and 200 m*M*. The results clearly demonstrated that diferric and monoferric transferrins and apotransferrin were eluted at 40, 90 and 200 m*M* sodium chloride in the acetate buffer (pH 5.4).

To determine whether the three distinct peaks (Fig. 2) separated on the Mono-S column represented transferrin with different types of iron binding, the individual fractions were characterized by 6 *M* urea–polyacrylamide gel electrophoresis (Fig. 1). Unfractionated transferrin could be resolved into four bands, in agreement with results of previous reports [3,7]. Analysis of the three individual HPLC-separated transferrin peaks revealed four bands, corresponding in mobility to four bands of pure transferrins with various degrees of iron content. In previous studies, transferrin could not be separated into monoferric transferrin from pure transferrin in native conditions.

The HPLC method used here allows the separation of monoferric transferrin under non-denaturing conditions from a mixture of transferrins with various degrees of iron content. A-site and B-site transferrins of the two monoferric

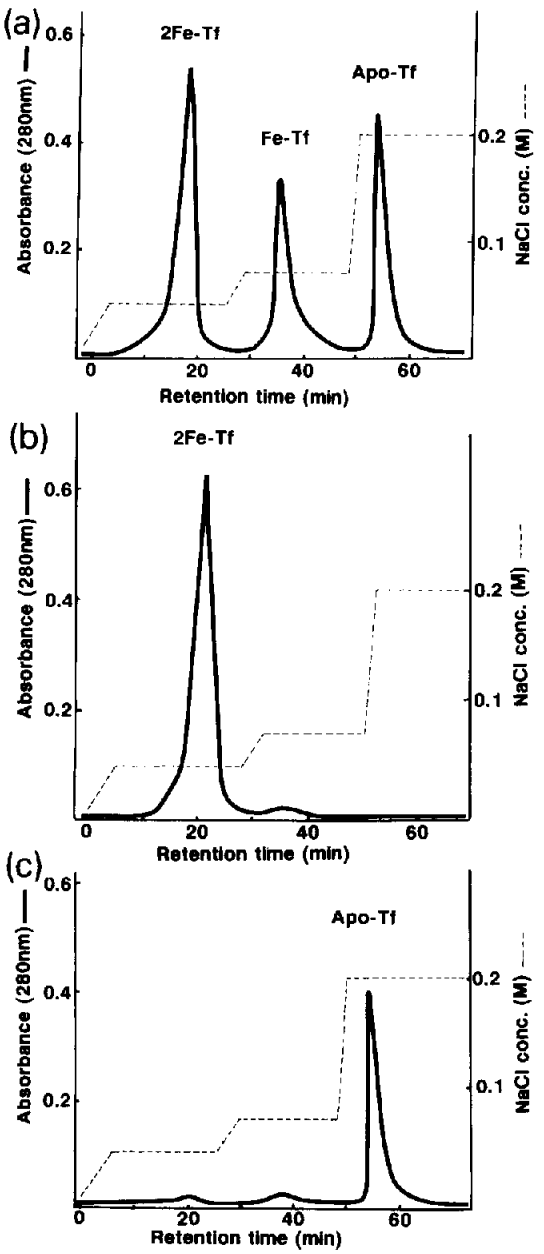

Fig. 2. Ion-exchange chromatographic separation of transferrin on a Mono-S HR 5/5 column (5 cm × 0.5 cm I.D., 10 μm) using a stepwise concentration gradient program of sodium chloride (40, 90 and 200 mM) in 0.02 M acetate buffer (pH 5.4). Flow-rate, 0.5 ml/min; detection wavelenght, 280 nm. Amount of sample applied: (a) 2.2 mg; (b) 1.2 mg; (c) 0.8 mg. Peaks: 2Fe-Tf = diferric transferrin; Fe-Tf = monoferric transferrin; Apo-Tf = apotransferrin. (a) Approximately 60% iron saturation transferrin; (b) diferric transferrin; (c) apotransferrin.

transferrin species were not distinguished under our experimental conditions. An analytical Mono-Q HR anion column did not separate individual transferrins.

The data presented in this paper indicate that the automated HPLC system with a Mono-S cation-exchange column can be suitably adapted for the detection and recovery of transferrins with different types of iron binding.

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REFERENCES

1 J. Fletcher and E. R. Huehns, *Nature*, 218 (1968) 1211.
2 P. Pootrakul, A. Christensen, B. Josephson and C. A. Finch, *Blood*, 49 (1977) 957.
3 P. Aisen, A. Leibman and H. A. Reich, *J. Biol. Chem.*, 241 (1966) 1666.
4 R. V. Wenn and J. Williams, *Biochem. J.*, 108 (1968) 69.
5 H. G. Van Eyk, R. J. Vermoat and B. Leijnse, *FEBS Lett.*, 3 (1969) 193.
6 I. Tei, Y. Makino, I. Kanamaru and K. Konno, *Phys.-Chem. Biol.*, 24 (1981) 7 (in Japanese).
7 D. G. Makey and U. S. Seal, *Biochim. Biophys. Acta*, 453 (1976) 250.
8 A. Leibman and P. Aisen, *Blood*, 53 (1979) 1058.
9 F. W. Putman, in F. W. Putman (Editor), *The Plasma Proteins*, Vol. 4, Academic Press, Orlando FL, 1984, p. 46.
10 I. Kanamaru, Y. Makino and K. Konno, *Phys.-Chem. Biol.*, 29 (1985) 205 (in Japanese).
11 H. Stibler and S. Borg, *Alcohol. Clin. Exp. Res.*, 10 (1986) 61.
12 K. Narita, I. Kanamaru, Y. Makino and K. Konno, *Clin. Chim. Acta*, 170 (1987) 85.
13 S. Petren and O. Vesterberg, *Biochim. Biophys. Acta*, 994 (1989) 161.
14 D. C. Harris, G. A. Gray and P. Aisen, *J. Biol. Chem.*, 249 (1974) 5261.
15 J. H. Jandl and J. H. Katz, *J. Clin. Invest.*, 42 (1963) 314.
16 G. W. Bates, C. Billups and P. Saltman, *J. Biol. Chem.*, 242 (1967) 2810.