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Note

Separation of asymmetrical hybrid hemoglobins by hydrophobic interaction chromatography

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Asymmetrical hybrid hemoglobins (Hb) are formed from two unlike $\alpha\beta$ dimers when two or more species of hemoglobin, either two different hemoglobins or two different states of the same hemoglobin, coexist in solution [1,2]. Although the liganded form of asymmetrical hybrid hemoglobins containing mutant hemoglobins, such as Hb Richmond and Hb Manitoba, can be isolated [3,4], it has been extremely difficult to isolate the liganded forms of most other hybrid hemoglobins including those containing Hb A, Hb S, and Hb C, because these hybrids dissociate quickly into $\alpha\beta^X$ dimers and then reassociate to form the original parent hemoglobins [1,2]. Previously, the separation of hybrid hemoglobins could be demonstrated only by converting hemoglobin to a more stable deoxy "T" structure [2,5,6] and by performing separation at low temperatures [7–9] or by using cross-linking reagents [10–12].

During experiments using gel-permeation chromatography of various hemoglobins on a TSK gel-permeation column in high phosphate buffer [13], we made the serendipitous finding that the oxy form of SF hybrid hemoglobin could be separated if the hemoglobins were eluted by 1.8 *M* potassium phosphate buffer, pH 7.4. This paper describes the first successful chromatographic separation of the oxy form of asymmetrical hybrid hemoglobin formed in a binary mixture of Hb F and Hb S (or Hb A) at room temperature.

EXPERIMENTAL

Hemoglobins A, F, and S were purified by chromatography on CM and DEAE Sephadex as described elsewhere [14,15]. The concentration of oxyhemoglobin

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was determined spectrophotometrically with the use of the millimolar extinction coefficient of $mA_{415}=125$ and $mA_{276}=34.4$ (as heme basis) [16]. Experiments were carried out using a Pharmacia fast protein liquid chromatographic (FPLC) system (Pharmacia, Piscataway, NJ, U.S.A.) composed of two P-500 pumps, an LCC-500 controller, a UV-1 monitor, and an MV-7 valve. The detectors were set at either 280 or 405 nm. The column used was a TSK-G-3000-SW (300×7.5 mm) purchased from LKB (Bromma, Sweden) [13]. For chromatography, 200 μl (ca. 100 μg) of hemoglobin was applied on the column using 0.1 and 1.8 *M* potassium phosphate buffer, pH 7.4, at room temperature with various flow-rates (0.1–1.0 ml/min).

We prepared deoxyhemoglobin by injecting an oxyhemoglobin solution into an anaerobic cuvette containing deoxygenated 1.8 *M* potassium phosphate buffer and sodium hydrosulfide (3 mg/ml). Oxyhemoglobins were converted to the deoxy form immediately. An aliquot of deoxyhemoglobin solution was applied on a TSK-G-3000-SW column equilibrated with deoxygenated 1.8 *M* potassium phosphate buffer containing 2 mg/ml sodium hydrosulfide, pH 7.4, at room temperature.

RESULTS AND DISCUSSION

As shown in Fig. 1A, if hemoglobins such as Hb A, Hb F, and Hb S were separated on a TSK-G-3000-SW column using 0.1 *M* potassium phosphate buffer, pH 7.4, as an eluent, they eluted as a single peak at the position where the molecular mass corresponds to approximately 60 000. In contrast, if the same experiments were carried out using 1.8 *M* potassium phosphate buffer, pH 7.4, each of these hemoglobins eluted differently even though their molecular masses were the same (Fig. 1B). We found that under this condition proteins eluted according to differences in the mode of hydrophobic interaction between the proteins and resin matrix [13]. Thus, we assumed that these hemoglobins could be separated if mixtures of these hemoglobins were subjected to chromatography using 1.8 *M* potassium phosphate buffer, pH 7.4, as an eluent. Unexpectedly, upon chromatography of 1:1 mixtures of oxy-Hb F and oxy-Hb S, three peaks instead of two were observed (Fig. 1C). The first and the third peaks represented Hb F and Hb S, while the identity of the hemoglobin in the middle peak was unknown.

To identify the hemoglobin in the middle peak, we first measured the absorption spectrum to determine if the hemoglobin had been oxidized or converted to another liganded or to an unliganded state. The spectrum was typical of oxyhemoglobin and indistinguishable from those of the two parent hemoglobins. Cellulose acetate electrophoresis of the CO form of hemoglobin in the middle peak showed two identically sized bands at the positions of Hb F and Hb S. From this, we concluded that the hemoglobin in the middle peak was the oxy form of asymmetrical FS hybrid hemoglobin ($\alpha_2\beta^F\beta^S$). Similar experiments with a 1:1 mixture of Hb A and Hb F also showed three peaks, with a hybrid hemoglobin peak between the peaks of the parent hemoglobins. To further confirm that the middle peak represented hybrid hemoglobin, we performed similar experiments after converting these hemoglobins to the deoxy form, which is more stable than the oxy form. As shown in Fig. 1D, the chromatogram of a 1:1 mixture of the deoxy

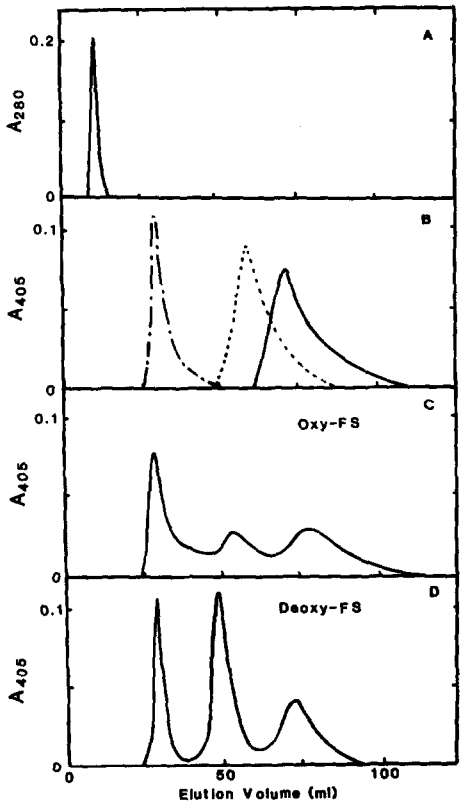

Fig. 1. Chromatograms of hemoglobin chromatographed at room temperature in a TSK-G-3000-SW. (A) Oxyhemoglobins A (– – –), S (---), and F(– · –) in 0.1 *M* potassium phosphate buffer, pH 7.4, with a flow-rate of 1 ml/min; (B) oxyhemoglobins A, S, and F in 1.8 *M* potassium phosphate buffer, pH 7.4; (C) oxy form of a 1:1 mixture of Hb F and Hb S in 1.8 *M* potassium phosphate buffer, pH 7.4; (D) deoxy form of a 1:1 mixture of Hb F and Hb S in 1.8 *M* potassium phosphate buffer, pH 7.4.

form of Hb F and Hb S revealed three peaks at a ratio of 1:2:1. Hemoglobin in the middle peak was converted to the CO form and subjected to cellulose acetate electrophoresis after dialysis with 0.1 *M* phosphate buffer, pH 7.0. Again, the hemoglobin in the middle peak produced two bands of equal size at the positions of Hb F and Hb S, indicating that the hemoglobin in the middle peak is the hybrid hemoglobin.

Stability of hybrid hemoglobins

The percentages of hemoglobin in the middle peak representing oxy forms of FS and FA hybrid hemoglobins to the total hemoglobin were 23 and 35%, respectively, when 1:1 mixtures of Hb F and Hb A or Hb S were chromatographed at a flow-rate of 1 ml/min. We found that the percentages of hybrid hemoglobins decreased when the elution time was prolonged. For instance, the middle peaks of Hb F–Hb A (FA) and Hb F–Hb S (FS) mixtures were significantly smaller or almost inapparent when the flow-rates were decreased three-fold. The relation-

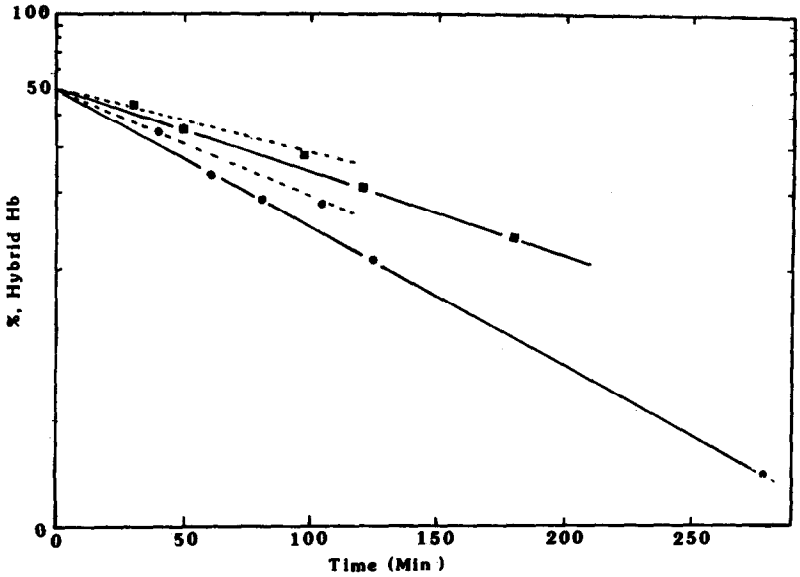

Fig. 2. Semilogarithmic plots representing the time course of the dissociation reactions of FA and FS hybrid hemoglobins. The percentages of FA and FS hybrid hemoglobin are plotted against the elution time. (■) Oxy form of FA hybrid hemoglobin; (●) oxy form of FS hybrid hemoglobin. The solid and dotted lines are the experiments at room temperature (22°C) and 6°C, respectively.

ship between the elution time and the percentages of hybrid hemoglobin is shown in Fig. 2. Semilogarithmic plots of the hybrid yielded from the mixture versus the elution time showed straight lines (Fig. 2). Extrapolation of the lines to time zero yields a value of approximately 50%, indicating that the percentage of hybrid hemoglobin at time zero is 50%. It appears that FS hybrid hemoglobin is more unstable than the FA hybrid, because the slope of the latter is greater than that of the former. Under the conditions of chromatography at room temperature, the half-lives of FS and FA hybrid hemoglobins are 53 and 93 min, respectively.

The effect of temperature on the stability of hybrid hemoglobins was also studied. Since the force for hydrophobic interaction is entropic, the interaction between hemoglobin and gel matrix should weaken at lower temperatures [13]. We found that each peak eluted faster and closer together at lower temperatures, suggesting that hydrophobic interaction is actually involved with the separation. However, the percentage of the hybrid hemoglobin increased at low temperatures as shown by the dotted line in Fig. 2, probably because of the decrease in the dissociation constant and of the increased flow-rate at lower temperatures.

It is known that the chromatographic behavior and the solubility of proteins are strongly affected by the surface properties of macromolecules and the concentration and chemical nature of the salt solution [17]. In low salt solutions, the mode of electrostatic interaction between the hydrophilic groups of proteins and ionic groups of resin plays a major role in the separation of proteins by ion-exchange chromatography. In high salt solutions, particularly in solutions containing salting-out anions such as phosphate or sulfate, proteins are separated by

differences in hydrophobic interaction [17]. Tanford [18] evaluated the total hydrophobicity of a protein by summing up the hydrophobicities of all amino acid residues. Melander and Horvath [17], who found that there was an inverse relationship between relative surface hydrophobicity and charge frequency, pointed out that the solubility and chromatographic behavior of proteins should depend on the hydrophobic properties of the surface residues. Results shown in this paper suggest another important factor. When dissociable proteins such as hemoglobin, which is in tetramer–dimer equilibrium, are chromatographed, dissociation constants as well as the surface properties of the intersubunit contacts of proteins affect the elution pattern. Also important is that the dissociation rate of tetrameric hemoglobin into $\alpha\beta$ dimers in high phosphate buffer appears to be much slower than that in low phosphate buffer.

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