Journal of Chromatography, 428 (1988) 247–254
Biomedical Applications
Elsevier Science Publishers B.V., Amsterdam — Printed in The Netherlands

CHROMBIO 4212

EFFECTS OF DIFFERENCES IN CHARGE AND HYDROPHOBICITY OF SURFACE AMINO ACIDS OF HEMOGLOBINS ON HIGH-PERFORMANCE GEL-PERMEATION CHROMATOGRAPHY

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(First received January 13th, 1988; revised manuscript received March 14th, 1988)

SUMMARY

We studied the elution properties of the carboxy and deoxy forms of hemoglobins A, S, and C in gel-permeation high-performance liquid chromatography using TSK-GEL-SW-type columns. Since these hemoglobins have the same molecular mass but different amino acids at the $\beta 6$ position, they are ideal for studies of the effect of charge and hydrophobicity on elution patterns in high-performance gel-permeation chromatography. Although there was a linear relationship between elution volume and logarithm of molecular mass of various proteins, the elution volumes of carboxyhemoglobins were found to be slightly greater than the expected volumes calculated from the molecular mass. The elution volumes of hemoglobins increased in the order of hemoglobins F, A, C, and S in 0.1 *M* phosphate buffer, pH 7.4, at room temperature. The elution volume of these hemoglobins was also dependent on pH and salt concentration. These results indicate that elution of these hemoglobins was affected by the electrostatic and hydrophobic interactions between hemoglobin molecules and polar sites of silica gel (with silanol groups) of the resin matrix of TSK-G2000-SW. This study may serve as a useful reference for separation and determination of molecular masses of proteins in the native state using gel-permeation liquid chromatography.

INTRODUCTION

High-performance gel-permeation liquid chromatography is frequently used to separate proteins and determine their molecular masses. Ideally, solute molecules are fractionated according to size because of exclusion from pores within the matrix. However, it is known that electrostatic interactions between protein molecules and the matrix can cause non-ideal size-independent elution [1,2]. We reported previously [3] that hydrophobic interaction between protein molecules and gel matrix changed the elution pattern of proteins if the high-ionic-strength

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mobile phase was used. To evaluate the effect of the hydrophobic and electrostatic interactions between proteins and gel matrix on elution patterns, we studied the elution behavior of hemoglobins A, S, and C. These hemoglobins not only have the same molecular mass but have amino acids with different charges and hydrophobicities at the 6th position of the β-chain on the surface of the molecule.

EXPERIMENTAL

Hemoglobins F, A, S, and C were purified by chromatography on CM and DEAE Sephadex [4]. The concentration of carboxyhemoglobins was determined spectrophotometrically with the use of the millimolar absorptivity of $mA_{569}=13.4$ (as heme basis) [5]. Calibration kit for molecular mass determination (ribonuclease A, chymotrypsinogen A, ovalbumin and aldolase) was purchased from Pharmacia Fine Chemicals (Uppsala, Sweden). A TSK-G2000-SW column (300 mm×7.5 mm I.D.), with a guard column for high-performance gel-permeation chromatography, was purchased from Bodman (Media, PA, U.S.A.). Chromatography was carried out on equipment from Waters Assoc. (Milford, MA, U.S.A.). Analysis by high-performance liquid chromatography (HPLC) was done using a Waters Assoc. system consisting of two Model 6000A pumps, a Model 450 variable-wavelength detector, a U6K injector, a data module, and a 720 system controller. The detectors were set at 280 nm. A constant volume (200 μl) of hemoglobins at various concentrations was injected into the column.

RESULTS

Elution volume and molecular mass of protein

If there is no interaction between a stationary phase and proteins, a linear relationship would be expected between the logarithm of the molecular mass (M_r) and elution volume (V_e). The TSK-GEL-SW-type gel particles have shown high recoveries with a good correlation between elution volume and log M_r for the number of proteins investigated [1–3,6–10]. Solid line and closed symbols in Fig. 1 show the relationship between V_e and log M_r for albumin, ovalbumin, chymotrypsinogen A, ribonuclease A, and human carboxyhemoglobins A, C, F, and S obtained using a TSK-G2000-SW column in 0.1 M potassium phosphate buffer, pH 7.4, at room temperature. The elution volumes of carboxyhemoglobins are slightly greater than the expected volumes calculated from their molecular masses. It should be mentioned that hemoglobins with the same molecular mass have different elution volumes (Fig. 2). For example, the elution volumes of hemoglobins F, A, C, and S increased in that order (Table I). The elution volume of each hemoglobin is reproducible with a standard deviation of less than ± 0.006 ($n=5$). This difference cannot be explained simply by the differences in charge, since the isoelectric points of these hemoglobins increased in the order of hemoglobins A, F, S, and C. Therefore, other factors such as hydrophobic interaction with gel matrix in addition to differences in surface charge and molecular size alter the elution patterns of these hemoglobins.

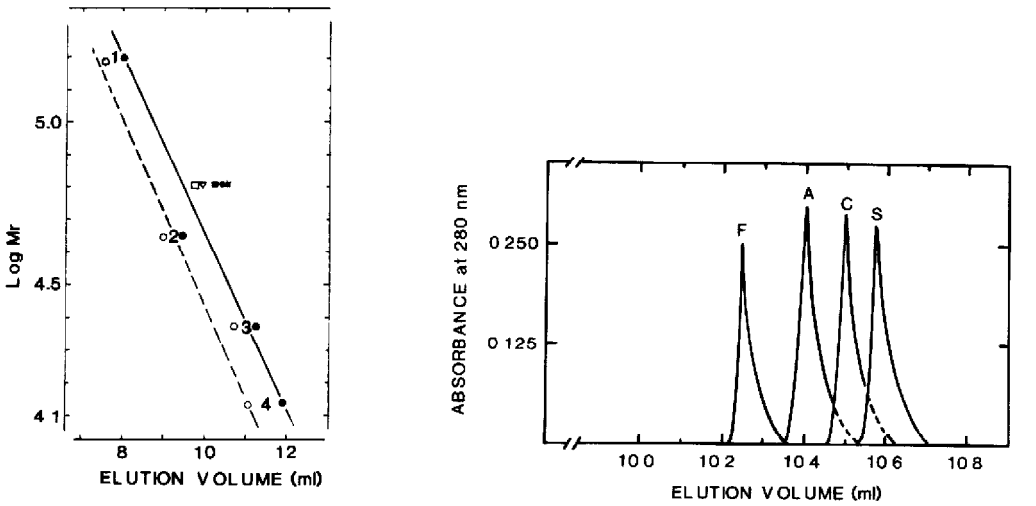

Fig. 1. Relationship between elution volume and logarithm of molecular mass. Solutions (200 μl) of carboxyhemoglobins (~250 μg) were injected on a TSK-G2000-SW column equilibrated with 0.1 *M* phosphate buffer, pH 7.4 or 7.0, in the absence or presence of 0.1 *M* sodium chloride, and eluted at a flow-rate of 0 5 ml/min at room temperature using the same buffer. Solid line with closed symbols shows results obtained under the conditions of 0.1 *M* phosphate buffer, pH 7.4. 1, 2, 3, and 4 are albumin, ovalbumin, chymotrypsinogen A, and ribonuclease A, respectively (20 μg in 200 μl). ■, ●, ▲, and × are carboxyhemoglobins F, A, C, and S, respectively. Dotted lines with open symbols show results obtained under the conditions of 0.1 *M* phosphate buffer, pH 7.0, in the presence of 0.1 *M* sodium chloride. □ indicates carboxyhemoglobins, A, F, and C. ▽ indicates carboxyhemoglobin S.

Fig. 2. Chromatograms of carboxyhemoglobins A, C, S, and F. Solutions (200 μl) of carboxyhemoglobins (~250 μg) were injected on a TSK-G2000-SW column equilibrated with 0 1 *M* phosphate buffer, pH 7.4, and eluted at a flow-rate of 0.5 ml/min at room temperature using the same buffer.

TABLE I

ELUTION VOLUMES OF CARBOXYHEMOGLOBINS IN 0.1 *M* PHOSPHATE BUFFER, pH 7.4

The experimental conditions were the same as those described in Fig. 1. Elution volume of each hemoglobin is the mean value of five trial runs.

Hemoglobin	Elution volume (ml)
A	10.40 ± 0.003
F	10.25 ± 0.004
C	10.48 ± 0.003
S	10.57 ± 0.006

Effect of hemoglobin concentration on elution volume

It is known that the elution volume of liganded hemoglobins with molecular-sieve chromatography depends on hemoglobin concentration [11]. The main reason is that tetrameric hemoglobin dissociates into dimers at low concentrations [11]. Therefore, molecular-sieve chromatography has been used to determine the tetramer–dimer equilibrium constant of hemoglobin [11,12]. We studied the con-

centration dependency of various hemoglobins on the TSK-G2000-SW column equilibrated with 0.1 *M* phosphate buffer, pH 7.4, at room temperature. We found that elution volumes of deoxyhemoglobins were unchanged in the injected hemoglobin concentrations between 0.1 and 25 μM, but that they differed depending on the type of hemoglobin. Since these deoxyhemoglobins do not dissociate into dimers under these experimental conditions, different elution patterns should be attributed to differences in surface properties of hemoglobin. In contrast, the elution volume of carboxyhemoglobins depended not only on higher concentration but also on the type of hemoglobin. By decreasing injected hemoglobin concentrations to less than 5 μM, the peak of elution volumes increased exponentially (Fig. 3). The elution volumes of carboxyhemoglobins increased in the order of hemoglobins F, A, C, and S, the same order as seen in deoxyhemoglobins. The elution volume of each carboxyhemoglobin at concentrations higher than 20 μM was larger than that of each deoxyhemoglobin, suggesting that the molecular mass of carboxyhemoglobin is smaller than that of deoxyhemoglobin. This difference may be caused by the existence of dimeric carboxyhemoglobin as well as differences in charge between deoxy- and carboxyhemoglobins.

Effect of pH on the elution volume

The effect of pH on the elution volume of carboxyhemoglobins A, S, F, and C was studied using a TSK-G2000-SW column and 0.1 *M* phosphate buffer, pH 6.5–7.5 (Fig. 4). The elution volumes of all four hemoglobins studied were minimal at around pH 7.0 and could be increased by either increasing or decreasing the pH. At pH 7.4, the elution volumes of hemoglobins increased in the order of hemoglobins F, A, C, and S. However, at pH values lower than 7.0, the elution volume increased in the order of hemoglobins F, A, S, and C, which corresponds to differences in charge in these hemoglobins. These data suggest that the con-

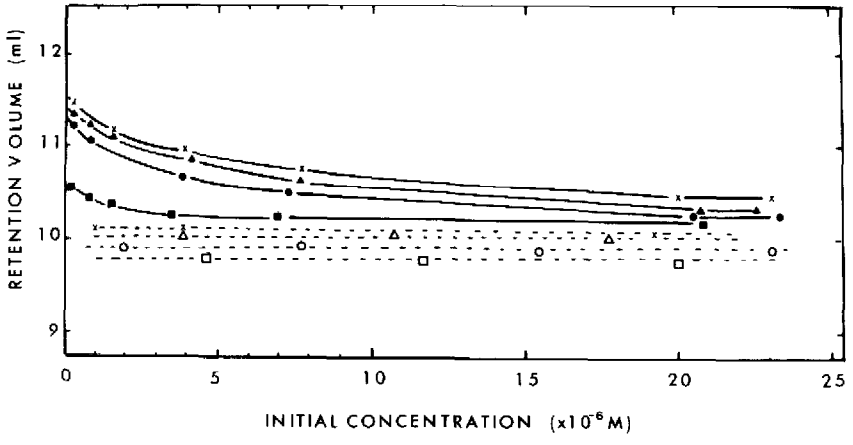

Fig 3. Relationship between elution volume and hemoglobin concentration on TSK-G2000-SW columns. Chromatographic conditions were the same as those described in Fig. 2 except for hemoglobin concentration. ×, ▲, ●, and ■ with solid lines indicate carboxyhemoglobins S, C, A, and F, respectively. ×, △, ○, and □ with dotted line indicate deoxyhemoglobins S, C, A, and F, respectively.

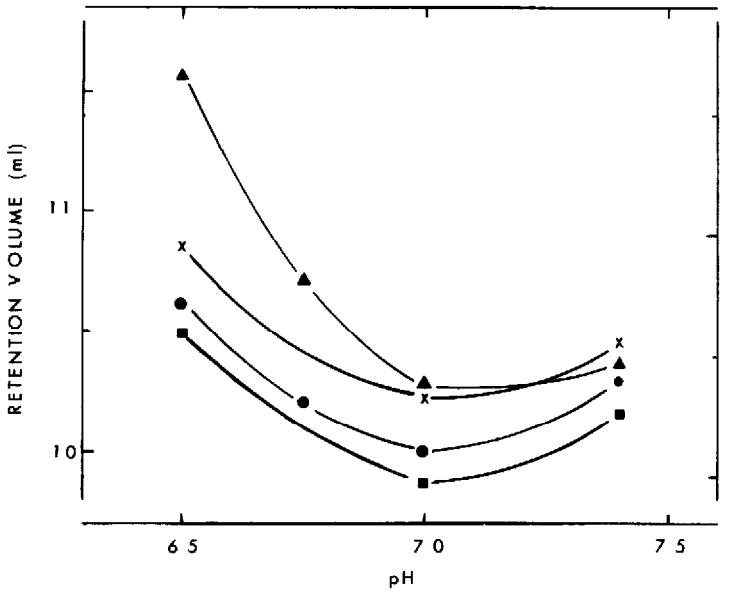

Fig. 4. Relationship between elution volume of hemoglobins and pH of mobile phase. Solutions (200 μl) of carboxyhemoglobins (~200 μg) were applied on a TSK-G2000-SW column and eluted by 0.1 *M* phosphate buffer with different pH at a flow-rate of 0.5 ml/min at room temperature. ■, ●, ▲, and × indicate carboxyhemoglobins F, A, C, and S, respectively.

tribution of surface charge of hemoglobins to elution volume could be increased by decreasing the pH of the elution buffer. In contrast, the hydrophobic interaction between hemoglobin molecules and the gel matrix appears to play a major role in separation at pH values higher than 7.0.

Effect of salt concentration

Effect of salt concentration in the elution buffer on elution volume of hemoglobins A and C was studied. It was found that by increasing salt concentration, electrostatic interaction diminished [13]. As shown in Fig. 5, the relationship between the elution volume of hemoglobins and ionic strength of sodium chloride used for the elution buffer is indicated by a concave curve. The lowest value was at a salt concentration of 0.1 *M*. Under this condition, the elution volumes of the two hemoglobins were similar, suggesting a minimal effect on retention time of the charge at the β6 position. However, when ionic strength is increased, hydrophobic interaction increases, thereby causing the increase in retention volume [13].

Based on these experimental results, we separated various hemoglobins and proteins under conditions where there is minimal effect from ionic interaction between hemoglobin molecules and gel matrix equilibrated with 0.1 *M* phosphate buffer, pH 7.0, containing 0.1 *M* sodium chloride (dotted line and open symbols in Fig. 1). As expected, carboxyhemoglobins A, C, and F eluted at similar positions but carboxyhemoglobin S eluted slightly later. This difference in elution position may be caused by differences in hydrophobic interaction. It should be noted that the elution volumes of hemoglobins A, F, and C deviated from a line

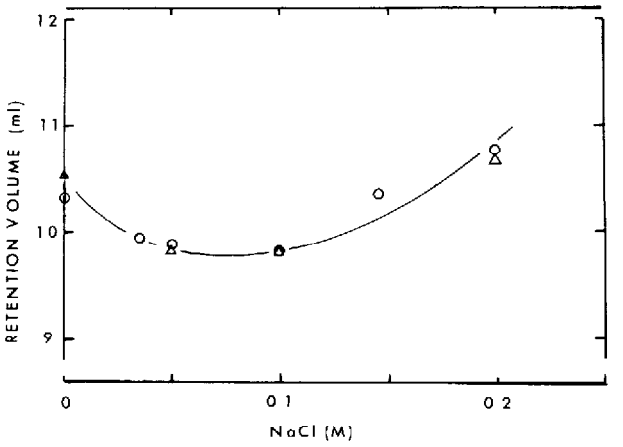

Fig. 5. Relationship between elution volume of hemoglobins and salt concentration. Solutions (200 μl) of carboxyhemoglobins A (○) and C (△) (~200 μg) were applied on the TSK-G2000-SW column and eluted at a flow-rate of 0.5 ml/min at room temperature. The mobile phase was 0.1 *M* phosphate buffer, pH 7.4, at various sodium chloride concentrations.

obtained using standard proteins. The molecular mass of these hemoglobins calculated from the line was about 56 000. The elution volumes of the deoxy form of these hemoglobins were the same as that of the carboxy forms under this condition, suggesting that the difference in elution volume between liganded and unliganded tetrameric hemoglobins in Fig. 3 is caused by differences in surface charge. These data indicate that deviations in molecular mass of hemoglobin in 0.1 *M* phosphate buffer, pH 7.0, containing 0.1 *M* sodium chloride can be predominantly attributed to the hydrophobic interaction between hemoglobin molecules and the stationary phase of gel.

DISCUSSION

Gel-permeation chromatography has been very useful in the isolation and purification of proteins and in the determination of their molecular masses. Two most commonly used columns for gel-permeation HPLC are the TSK-SW and SynChropak GPC types. TSK-SW series gel packings are particulate silica materials covalently bonded with hydrophilic compounds. The gel surface has negative charges that are probably due to traces of silanol groups that remain unreacted and consequently affected by the cation concentration in the elution buffers [14].

Our results clearly demonstrated that the elution patterns of hemoglobin on gel-permeation chromatography using the TSK-GEL-SW packing column is affected by pH and salt concentration of the buffer in addition to dissociation of tetrameric hemoglobin into dimers. This effect becomes minimal in the presence of 0.1 *M* sodium chloride and at buffers of pH values near 7.0. Our present data also suggested that the polar surface of silica gel with silanol groups might interact with protein molecules. The mode of interaction may differ, depending on the

type of protein and elution conditions. Positively charged hemoglobin C interacted strongly with the gel matrix at lower ionic strengths and lower pH values. In contrast, at higher pH values and at high salt conditions, the difference in the elution volume between hemoglobins A and C diminished. We also found that at phosphate concentrations above 1 *M*, hemoglobins A and C could be separated on a TSK-GEL-SW column [3]. Under such conditions, this column acts as a hydrophobic-interaction column rather than as a gel-permeation column. It is known that an increase in salt concentration decreases electrostatic interaction [13]. Our present results on the elution behavior of hemoglobins clearly demonstrated this. The increase in salt concentration decreases surface charges of hemoglobin molecules, causing decreases in electrostatic interaction and increases in hydrophobic interaction.

The application of gel-permeation HPLC to proteins [15–17] has been well documented. It is generally recognized that these columns provide several distinct advantages over the conventional soft-gel-permeation columns such as Sephadex and Bio-Gel, namely their significant reduction in both analysis time, sample size, and band spreading. As to the determination of molecular masses of hemoglobin molecules with soft gel, it is recognized that the deviation is caused by the tetramer–dimer dissociation of hemoglobin molecules, because estimates are based on the standard calibration curve derived from globular protein [11,12]. In contrast, the non-ideal behavior of hemoglobin on high-performance gel-permeation chromatography not only depends on tetramer–dimer dissociation but also on specific electrostatic and hydrophobic interactions between hemoglobin molecules and the stationary phase of the gel. Thus, when gel-permeation HPLC is used for the determination of the molecular mass of proteins in native states the factors shown in this paper have to be considered.

ACKNOWLEDGEMENTS

The authors wish to thank Janet Fithian for editorial assistance. This work was supported by Research Grants HL-20750 and HL-32908 from the National Institutes of Health.

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