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BEHAVIOUR IN A SILICA-BASED HIGH-PERFORMANCE LIQUID GEL PERMEATION CHROMATOGRAPHIC COLUMN OF THE APO- AND HOLO-FORMS OF THE HAEM-BINDING PROTEINS HAEMOPEXIN, HISTIDINE-RICH GLYCOPROTEIN, GLOBIN AND ALBUMIN

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SUMMARY

The elution of the apo- and holo-forms of four haem-binding proteins was studied using a TSK G 3000 SW HPLC column. Apo-haemopexin had a higher apparent molecular size [68 000 daltons (d)] than histidine-rich glycoprotein (HRG) (66 000 d) in gel chromatography, contrasting with the values in sodium dodecyl sulphate electrophoresis, 84 000 d for HRG and 69 000 d for haemopexin. The elution of the haem complexes of both proteins correlated better with their true molecular weights. Saturation of albumin with haem did not significantly influence its elution. The peaks were more symmetrical for the holo- than the apo-proteins, except for globin/haemoglobin. The results indicated that the apo-forms of haemopexin and HRG had affinity for the column matrix. HRG, which has several haem binding sites, was retained more than haemopexin, which binds only one haem. Free haem itself was bound to the silica column but could be released by globin. HRG had a tendency to polymerize after haem binding, in contrast to haemopexin, which remained monomeric. Globin was eluted from the column with an apparent molecular size of 16 000 d and after saturation with haem with a molecular size of 31 000 d.

INTRODUCTION

High-performance liquid chromatography (HPLC) in gel permeation columns rapidly separates proteins differing in molecular size. However, the results of molecular weight determinations by this technique are not always reliable because all proteins are not globular and some proteins interact with the column matrix. Also, a single elution buffer is not suitable for all proteins[1–3].

The chemically modified silica matrix of a new TSK G 3000 SW column usually has a low affinity for proteins and the recoveries are mostly above 95%[4,5]. However, with use, new silica groups are generated by erosion, which increases undesirable interactions, ion exclusion, cation exchange and hydrophobic interactions[6]. It is possible to reduce the ionic interactions by increasing the ionic strength of the elution buffer, but at the same time hydrophobic interactions increase[3]. Special problems

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have been posed by very cationic and anionic proteins or ligand-binding and complex-forming proteins[7]. Ligand binding may alter the conformation of the apo-protein, resulting in a changed Stokes radius or polymerization of the protein[8,9].

In this study, the effect of haem on the elution behaviour of haem-binding proteins was evaluated. Preliminary eperiments showed that the apo- and holo-forms of haemopexin had different elution volumes, and a study was initiated to determine whether the phenomenon was due to a change in Stokes radius or different affinities of the apo- and holo-forms for the column matrix. A new method was developed for the separation of haem–protein complexes from free haemin and apo-protein, based on the use of a Haem-Sepharose affinity column which binds free haemin and apo-protein but not haem–protein complexes.

EXPERIMENTAL

Isolation of haemopexin and histidine-rich glycoprotein (HRG)

The Haem-Sepharose affinity gel was synthesized as described by Majuri *et al.*[10]. Hog serum was diluted twice with 0.1 *M* phosphate buffer (pH 7.0) and filtered through a 0.22-μm Durapore membrane filter (Millipore). Haem-Sepharose affinity gel (10 ml) was added to the diluted serum and the mixture was incubated in a slowly rotating mixer overnight at 4°C in the presence of 0.1% phenylmethylsulphonyl fluoride (PMSF) and 0.01% sodium azide.

The gel was packed into a small column and washed with 0.1 *M* phosphate–0.1 *M* sodium chloride (pH 7.2) (2000 ml) and finally with distilled water (200 ml). Adsorbed proteins were eluted with 0.02 *M* citric acid and the eluate was immediately neutralized with Na_2HPO_4 and finally dialysed against 0.02 *M* sodium phosphate buffer (pH 6.5).

The eluate was then applied to a Mono S HPLC column (Pharmacia), equilibrated with 0.02 *M* sodium phosphate buffer (pH 6.5). Both haemopexin and HRG were adsorbed on the column. Pure haemopexin was eluted from the column by increasing the sodium chloride concentration of the eluting buffer to 0.25 *M* and HRG with 1 *M* sodium chloride solution. To release non-covalently bound haem the Haem-Sepharose column was regenerated by washing with 20% pyridine in water.

Isolation of globin

Haemoglobin and globin were purifed from outdated human red cell concentrate (Finish Red Cross Transfusion Service, Helsinki, Finland) by the method of Winterhalter and Huehns[8], using CM-Sephadex ion-exchange chromatography and acetone–hydrochloric acid precipitation at −20°C.

High-performance liquid chromatography

Gel permeation runs were performed with a new LKB Ultrapac TSK G 3000 G column (600 × 7.5 mm I.D.). The number of theoretical plates of 35277 and an asymmetry factor of 1.31 were given by the manufacturer for the column and were calculated for the ethylene glycol peak. The HPLC apparatus consisted of a Rheodyne 7125 injector with a 200-μl capillary loop, LKB 2150 HPLC pump and LKB 2158 Uvicord SD detector with a 280-nm filter and an LKB 2210 recorder. The runs were performed at room temperature in 0.1 *M* sodium phosphate–0.1 *M* sodium

chloride (pH 7.2) buffer with a flow-rate of 0.5 ml/min. Samples were passed through a 0.45-μm HV filter (Millipore).

Molecular weight standards

Standards were dissolved in the HPLC running buffer to produce a concentration of about 0.1 mg/ml. A calibration graph was prepared using the elution volumes of rat [^{125}I]haemoglobin–haptoglobin complex [162000 daltons (d)], human IgG (160000) and serum albumin (68 000 d) (Finnish Red Cross Transfusion Service), ovalbumin (43000 d), myoglobin from equine skeletal muscle (17500 d) and cytochrome C from horse heart (12270 d), all from Sigma. The totally excluded volume of the column (V_0) was determined from the peak of polymerized IgG and the total volume (V_t) from the elution peak of cyanocobalamin (1355 d).

Saturation of proteins with haem

Haemin (type III, Sigma) was dissolved in 0.1 *M* sodium hydroxide solution and neutralized with 0.1 *M* sodium phosphate (pH 7.0) and filtered through a 0.22-μm Durapore membrane. Haemopexin, HRG, albumin or globin were incubated in a rotating mixer with saturating concentrations of haemin for 1 h at room temperature. The samples were then applied to a 28 × 6 mm I.D. column of Haem-Sepharose. Free haemin and the apo-proteins were adsorbed on the column, but the haem–protein complexes passed through as visible coloured peaks, which were collected. In some experiments DEAE-Sepharose was used to remove excess of haem as described earlier[11].

Asymmetry factor

Asymmetry factors (*As*) for the protein peaks in the gel permeation chromatograms were determined by the one-tenth peak-height method ($As = b/a$), where h is peak height and a and b are the half-widths of a peak at one tenth of the peak height.

Other methods

Sodium dodecyl sulphate (SDS) gel electrophoresis in 7.5 or 12% polyacrylamide slab gels (SDS-PAGE) was performed according to the method of Laemmli[12]. Molecular weight markers were human albumin, human transferrin (Sigma), ovalbumin and haemoglobin. Protein was determined by the method of Lowry *et al.*[13] with human serum albumin (Kabi) as a standard. Absorption spectra of haem–protein complexes were determined with a Beckman Model 25 recording spectrophotometer. Hog haemopexin was identified by its capacity to cross-react with anti-human haemopexin immune serum (Kabi) and HRG from its spectrum. Isoelectric points were determined for ^{125}I-labelled hog haemopexin and HRG by focusing in preparative columns using Pharmacia ampholytes.

RESULTS AND DISCUSSION

HRG has now been isolated from hog serum for the first time. The protein is composed of one polypeptide chain with a molecular weight of 84000 d in SDS electrophoresis. However, in the TSK G 3000 SW column it could not be separated

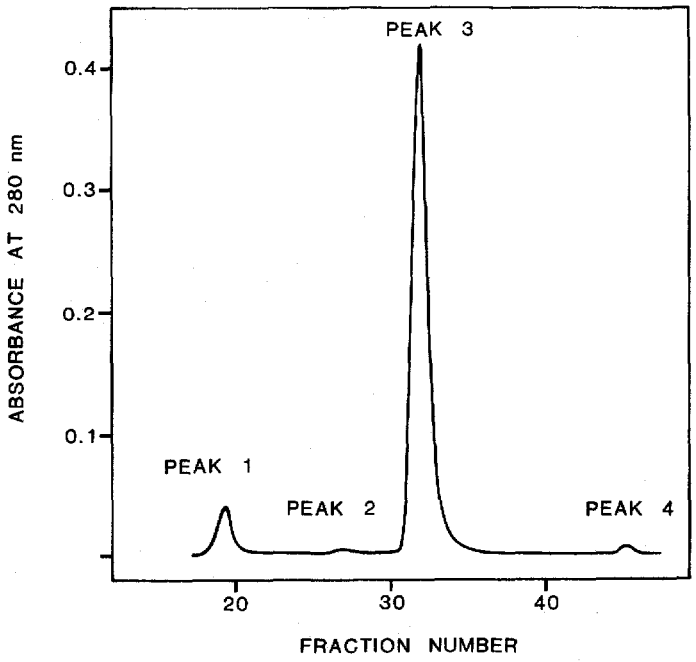

Fig. 1. Results showing that hog haemopexin and HRG did not separate in the TSK 3000 SW column and were contained in peak 3. Peak 2 is the dimer of HRG; peak 1 is totally excluded aggregated protein. The proteins in peaks 1 and 4 remained unidentified.

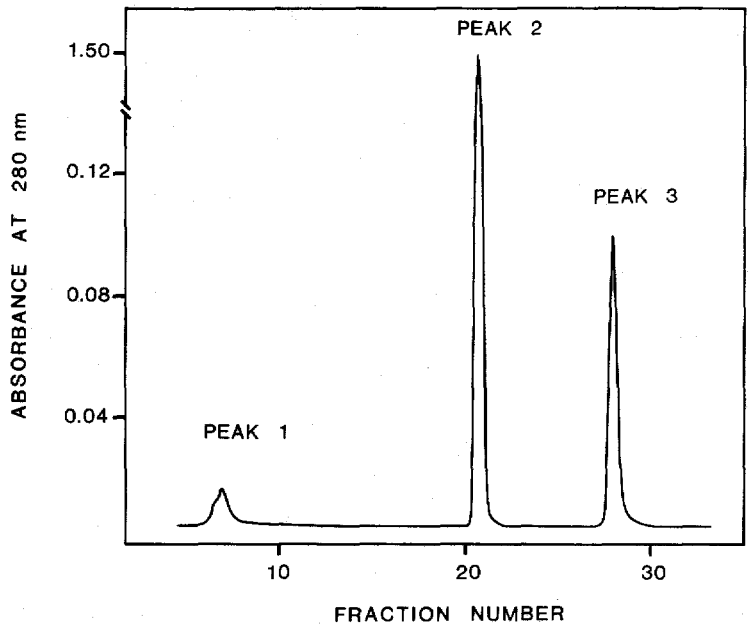

Fig. 2. Separation of hog haemopexin (peak 2) and HRG (peak 3) with a Mono S column. Both proteins were homogenous in SDS-PAGE. The protein in the breakthrough fraction (peak 1) was not identified.

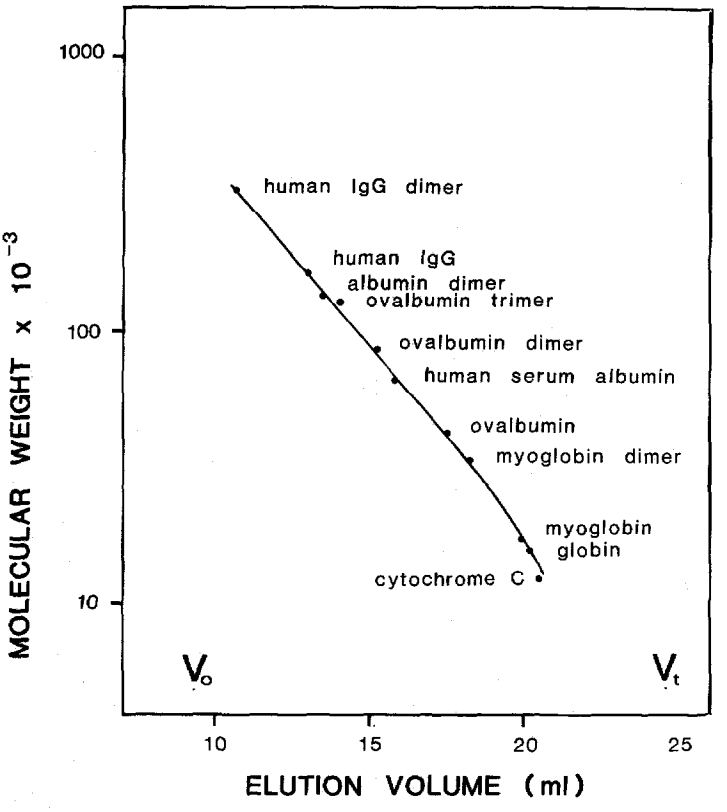

Fig. 3. Retention time *versus* molecular weight plot for standard proteins. The running buffer was 0.1 *M* sodium phosphate–0.1 *M* sodium chloride (pH 7.2), flow-rate 0.5 ml/min. Samples (200 µl) were run at room temperature and the eluate was monitored by absorption at 280 nm.

from the other, more abundant haem-binding protein haemopexin, which in SDS electrophoresis seemed to have a molecular weight of 69 000 d (Fig. 1). These proteins have very different isoelectric points and the Mono S cation-exchange HPLC column easily separated them, as seen in Fig. 2. As the Soret band was not visible, the haem-binding proteins were eluted from the Haem-Sepharose affinity column without attached haem.

Next, the possible effect of haem on the elution volumes of haemopexin and HRG was studied in a new TSK G 3000 SW column. First the column was calibrated with known marker proteins (Fig. 3). Because in preliminary experiments the G 3000 SW column was observed to bind haem, it became necessary to remove excess free haemin after the saturation of the proteins. For this purpose a small column of Haem-Sepharose was found to be very convenient. As shown in Fig. 4, the column bound more than 10 mg of haemin. The same column also adsorbed protoporphyrin IX, but not as effectively as haemin, which was totally adsorbed, probably by polymerization with covalently bound haem.

Fig. 5A shows the elution peaks of pure apo-haemopexin and its haem complex. The former had an apparent molecular size of 68 000 d and the latter 74 000 d.

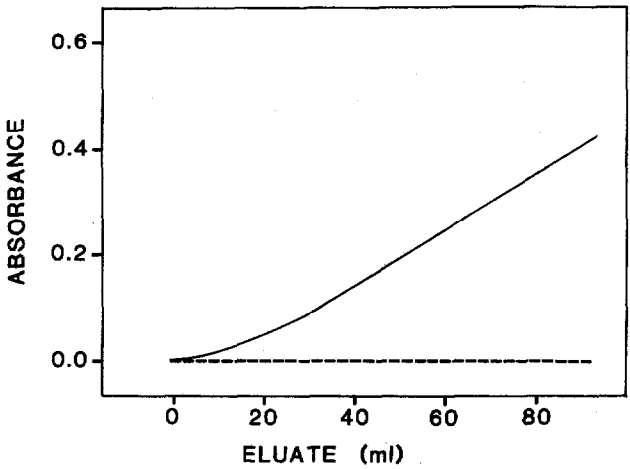

Fig. 4. Results for 10 mg of haemin or protoporphyrin IX in 100 ml of running buffer applied to a Haem-Sepharose column (28 × 6 mm I.D.). The absorbance was monitored at 395 nm for the detection of haemin (broken line) and at 420 nm for protoporphyrin IX (solid line). Haemin was totally adsorbed but protoporphyrin IX only partially.

In an old column the difference between the retention times of apo- and holo-haemopexin was even more evident (Fig. 5B). Saturation of haemopexin with haem did not cause the protein to polymerize and it still emerged as one peak. The peaks of the apo- and holo-proteins were so close to each other that they could not be separated if they were run together.

In its apo-form, HRG was only slightly dimerized (Fig. 6). However, incubation of the protein with haemin changed the elution pattern considerably. The position of the monomer peak changed to an apparent molecular size of 84 000 d, which is consistent with its molecular size in SDS electrophoresis (Table I). The monomer of the apo-protein emerged from the column indicating a molecular size of 66 000 d. The asymmetry factor (2.0) for the peak indicated tailing and affinity of the protein for the column. In contrast, the asymmetry factor for the peak of the holoprotein monomer (1.4) was very near the theoretical minimum for this column. HRG also polymerized in the presence of haemin, the amount of dimer and trimer being dependent on the duration of the incubation time with haemin. If the incubation was continued for several hours at room temperature, larger polymers appeared and the monomer peak disappeared.

Globin emerged from the TSK G 3000 SW column with an apparent molecular size of 16 000 d, slightly later than myoglobin (17 500 d). Saturation of the globin with haem changed the elution position to an apparent molecular size of 31 000 d and increased the asymmetry factor of the peak from 1.16 to 2.75. This haemoglobin preparation emerged from a calibrated Sephacryl S-300 column according to the molecular size of 64 000 d, which is the molecular size of native haemoglobin. The result indicates that globin has no affinity for the G 3000 SW column but that haemoglobin has (Fig. 7). If the globin sample was injected into the column that had bound free haemin, it emerged as a triplet peak, corresponding to molecular sizes of 16 000, 26 000 and 31 000 d (results not shown). Globin was able to release matrix-bound

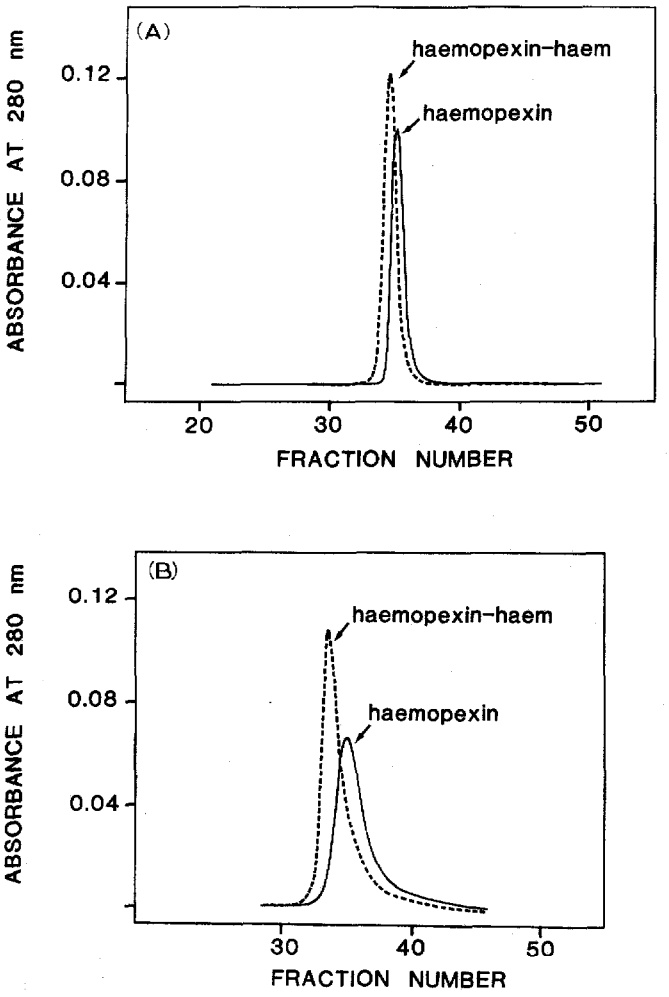

Fig. 5. (A) Results showing that hog apohaemopexin (solid line) and the haem complex of haemopexin (broken line) had slightly different elution volumes in a new TSK G 3000 SW column. (B) Results showing that the difference was more apparent if the same runs were performed in an old column.

haem and probably formed alpha–beta dimer and tetramer complexes. This run was useful for testing wether haem had contaminated the column. The retention times of serum albumin and haem–albumin (methaem–albumin) did not differ significantly and the apparent molecular sizes were 68 000 and 69 000 d (Table I).

Uncoated silanol groups of the silica columns have been found to interact electrostatically, especially with cationic proteins, resulting in reduced filtration rates, tailing and unexpectedly low recoveries[5]. Kato and Hashimoto[6] also found that high-pressure size-exclusion chromatography columns exhibit weak hydrophobic properties at high salt concentrations. The hydrophobicity of the proteins studied here is not high but hog HRG is a fairly cationic protein (isoelectric point 10.4) and has a

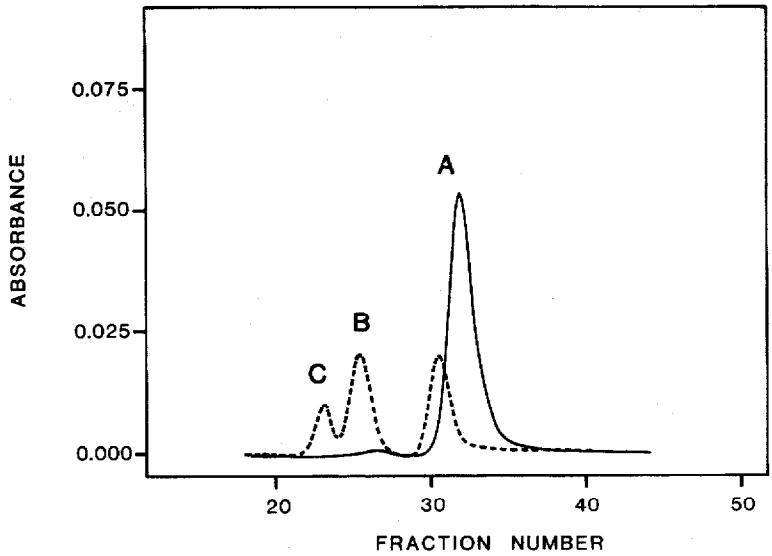

Fig. 6. Elution profiles of apo-HRG (solid line) and haem complex of HRG (broken line) in a TSK G 3000 SW run. The monomer peak of HRG (A) was eluted earlier if the protein was complexed with haem; dimer (B) and trimer (C) peaks also appeared.

positive charge in the present elution buffer. Its charge is partially neutralized after binding of several molecules of haem[14], which could explain the difference in the elution volumes of the apo- and holo-proteins. In contrast, hog apo-haemopexin has an isoelectric point of 5.8 and a negative charge in the running buffer (pH 7.2).

TABLE I

APPARENT MOLECULAR WEIGHTS OF HAEM-BINDING PROTEINS

The retention times of four haem binding proteins in the apo-form and after saturation with haem were compared in a TSK G 3000 SW column. The runs were performed in 0.1 *M* sodium phosphate–0.1 *M* sodium chloride buffer (pH 7.2) at a flow-rate of 0.5 ml/min. Apparent molecular weights were determined using a calibration graph for known marker proteins. The asymmetry factors for the elution peaks were calculated as described above. The same samples were also analysed by SDS-PAGE.

Protein	*TSK G 3000 SW column*			*SDS-PAGE: molecular weight*
	Retention time (min)	*Asymmetry factor*	*Molecular weight*	
Globin	40.4	1.2	16 000	16 000
Haemoglobin	37.0	2.7	31 000	16 000
Albumin	31.5	1.2	68 000	68 000
Haem–albumin	31.4	1.2	69 000	68 000
Haemopexin	31.5	1.4	68 000	69 000
Haem–haemopexin	31.1	1.3	74 000	69 000
HRG	31.9	2.0	66 000	84 000
Haem–HRG	30.5	1.4	84 000	84 000

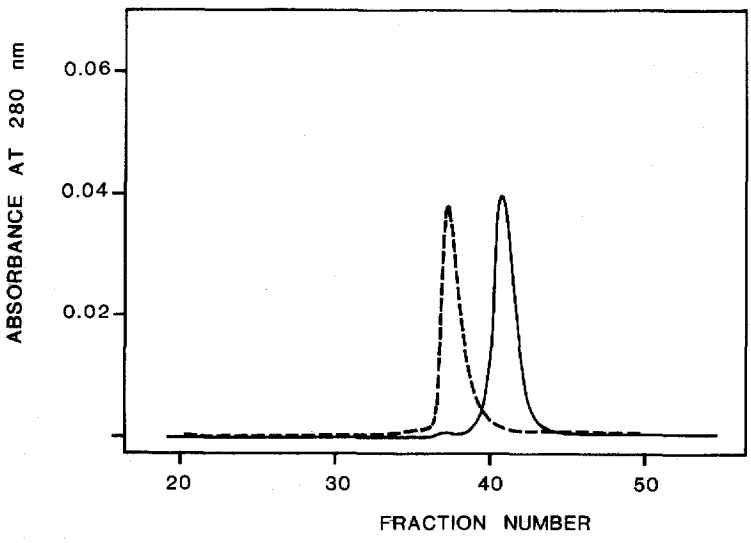

Fig. 7. Globin (solid line) and haemoglobin (broken line) runs in a TSK G 3000 SW column.

However, the apo-haemopexin was eluted later than expected from the difference in the true molecular weights of the apo- and holo-forms. Its elution behaviour can be explained by a conformational change of the protein after binding of haem. However, the difference noted between the results observed with an old and a new column indicates that a more likely explanation is higher affinity between apo-haemopexin and the column than the holo-haemopexin and the column. This assumption is also supported by increased tailing of the apo-protein peak. Whether the interaction is caused by the haem-binding site or by the surface charge remains an open question.

Globin was eluted as if its molecular size were 16000 d, which is the molecular size of the alpha and beta chains of haemoglobin. The small asymmetry factor indicated that the protein did not interact with the column matrix. However, as also noticed by others[15], both human and rat haemoglobin were retained. In contrast, the rat haemoglobin–haptoglobin complex had the expected elution volume (molecular weight 162000 d) and thus did not have an affinity for the column.

In conclusion, the apo-forms of the serum haem-binding proteins HRG, haemopexin and albumin have very similar elution volumes in the TSK G 3000 SW HPLC gel permeation column in spite of the differences in their true molecular weights. The elution positions of the monomer peaks are changed after haem binding and correlate better with their true molecular sizes than those of the apo-proteins. The haem complexes of these proteins can easily be separated from free haem using Haem-Sepharose affinity gel. Removal of haem is necessary before the runs in order to protect the columns from contamination with haem.

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