Journal of Chromatography, 327 (1985) 313–325
Elsevier Science Publishers B.V., Amsterdam — Printed in The Netherlands

CHROMSYMP. 573

DETERMINATION OF THE DISSOCIATION CONSTANT OF OLIGOMERIC PROTEINS BY SIZE-EXCLUSION HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY: APPLICATION TO HUMAN HAEMOGLOBIN

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SUMMARY

The measurement of protein retention volumes on a size-exclusion chromatographic column offers the possibility of determining dissociation constants for oligomeric proteins, as changes in the retention volume, depending on the concentration of the protein, are due to a dissociation equilibrium. The retention volume may be calibrated in terms of dissociation constant by using either extreme concentration conditions or chemical modifications that shift the equilibrium towards a single species. When zonal chromatography is used, the dilution during elution modifies the equilibrium state. In contrast, the saturation method permits the concentrations of the different species to be kept constant. These two methods were compared and the elution factor that must be used in zonal chromatography on high-performance size-exclusion columns (LiChrospher Diol) was obtained. The tetramer–dimer dissociation constants of normal and modified haemoglobins were measured by this method, and the results are in accordance with flash photolysis measurements.

INTRODUCTION

Size-exclusion chromatography has been used for the characterization of self-associating proteins. Following the theoretical predictions of Gilbert[1–4], the utility of this type of chromatography was illustrated by the qualitative study of self-association of α-chymotrypsin[5,6] and extended to the study of other protein associations[7]. Nichol *et al.*[8] described a method that takes into account all types of interactions between proteins to form rapidly reversible complexes.

In all these instances, the precise analysis of leading and trailing boundaries permits the determination of retention volumes for different species at equilibrium and the deduction of association constants. Brumbaugh and Ackers[9] introduced another technique for measuring the weight-average partition coefficient of different haemoblobins. In their technique, the column is saturated with the protein solution.

0021-9673/85/$03.30

By measuring the optical absorbance of a column section (gel and solution), penetration of the solute into the pores is obtained and used to calculate the association constant of the haemoglobin tetramer–dimer system. More refined techniques have been presented by the same groups[10,11].

In this paper, we introduce two methods for obtaining haemoglobin association parameters. The first is based on a saturation method, previously described[12] for drug–protein binding. The second uses classical zonal chromatography, applied to haemoglobin solutions at different concentrations. We applied the latter method to HbS and chemically modified haemoglobins with different reagents. Our results were compared with those obtained by a flash-photolysis method, previously described by Edelstein *et al.*[13] for carboxyhaemoglobin (Hb-CO), which was shown to be equivalent to sedimentation measurements.

THEORY

Considering the equilibrium between Hb tetramer T and Hb dimer D:

$$T \overset{^4K_2}{\rightleftharpoons} 2D$$

the retention volumes of the different species after size-exclusion chromatography are given by

$$V_T = V_0 + \sigma_T V_p \tag{1}$$

and

$$V_D = V_0 + \sigma_D V_p \tag{2}$$

where V_0 is the interstitial volume, V_p the pore volume and σ_T and σ_D are the permeation (or penetration) coefficient of the tetramer and dimer, respectively:

$$\sigma_T = \frac{[T]_p}{[T]_m} \tag{3}$$

$$\sigma_D = \frac{[D]_p}{[D]_m} \tag{4}$$

where [T] and [D] are the tetramer and dimer Hb molar concentration, respectively. The subscripts p and m indicate pore volume and mobile phase, respectively.

When the equilibrium kinetics are sufficiently rapid, the injected Hb sample emerges from the column as a unique peak with a retention volume situated between V_T and V_D and corresponds to a resulting chromatographic coefficient σ:

$$\sigma = \frac{[Hb]_p}{[Hb]_m} \tag{5}$$

where $[Hb]_p$ and $[Hb]_m$ are the total Hb molar concentration in each phase. Then,

$$[Hb]_p = [T]_p + \frac{[D]_p}{2} \tag{6}$$

and

$$[Hb]_m = [T]_m + \frac{[D]_m}{2} \tag{7}$$

Eqn. 5 becomes

$$\sigma = \frac{[T]_p + \frac{[D]_p}{2}}{[T]_m + \frac{[D]_m}{2}} \tag{8}$$

and from eqns. 3 and 4

$$\sigma = \frac{2\sigma_T\frac{[T]_m}{[D]_m} + \sigma_D}{2\frac{[T]_m}{[D]_m} + 1} \tag{9}$$

If σ is the fraction of dissociated haemoglobin in the mobile phase,

$$[T]_m = [Hb]_m (1 - \alpha) \tag{10}$$

and

$$[D]_m = 2\alpha[Hb]_m \tag{11}$$

Then

$$\frac{[T]_m}{[D]_m} = \frac{1 - \alpha}{2\alpha} \tag{12}$$

and from eqns. 9 and 12

$$\sigma = \frac{2\sigma_T(1 - \alpha) + 2\sigma_D\alpha}{2(1 - \alpha) + 2\alpha} = \sigma_T(1 - \alpha) + \sigma_D\alpha$$

$$\sigma = \sigma_T + \alpha(\sigma_D - \sigma_T) \tag{13}$$

After passage into the column, the Hb sample is eluted at a volume V such that

$$V = V_0 + \sigma V_p \tag{14}$$

From eqns. 13, 1 and 2 we obtain

$$\sigma = \frac{\sigma - \sigma_T}{\sigma_D - \sigma_T} = \frac{V - V_T}{V_D - V_T} \tag{15}$$

We have neglected the weak change in V_T and V_D induced by physical interactions with the gel, previously described by Windsor and Sheraga[5].

EXPERIMENTAL

Saturation method

In this method, the column is saturated with oxygenated haemoglobin [$HbA(O_2)$] solution, which flows continuously. The injection of a small volume (50 μl) of aqueous buffer leads to the appearance of a negative peak at the retention volume of the protein. The measurement of V gives α, as explained above, and as the Hb concentration C is known and constant during the elution, we obtain the dissociation constant ${}^4K_2$ of tetramer–dimer equilibrium by the law of mass action:

$${}^4K_2 = \frac{4C\alpha^2}{(1 - \alpha)} \tag{16}$$

Zonal chromatography

As the saturation method requires a large amount of protein, we also used typical zonal elution chromatography. In this instance there is a continuous change in the equilibrium during elution, owing to the dilution effect and to the different penetrations of Hb tetramer and dimer into the pores.

In this type of chromatography, there is a new equilibrium, which corresponds to a dissociated fraction α_{eq} of Hb, the value which is obtained from eqn. 15 from the measured elution volume. This equilibrium can be considered to result from the dissociation of an equivalent Hb solution saturating the column at the "equilibrium equivalent concentration", C_{eq}, defined by the law of mass action (eqn. 16):

$$C_{eq} = \frac{{}^4K_2(1 - \alpha_{eq})}{4\alpha_{eq}^2} \tag{17}$$

This saturating solution would have the same retention volume as the injected solution at concentration C_0.

If we consider the initial state of dissociation, α_0, then between C_0 and σ_0 we have an equation similar to eqn. 17. Then,

$$\frac{(1 - \alpha_0)\alpha_{eq}^2}{\alpha_0^2(1 - \alpha_{eq})} = \frac{C_0}{C_{eq}} = f \tag{18}$$

This quantity, named here the "elution factor", can be experimentally measured with $HbA(O_2)$ under different conditions (see below) and, as it is independent of ${}^4K_2$, it can be applied to different types of Hb. The value of f is a function of the measured

α_{eq} and is used to obtain C_{eq} from the value of C_0 (injected). From a knowledge of α_{eq} and C_{eq} for several concentrations injected, it is possible to determine $^4K_2$ for each haemoglobin.

HPLC

A 6000A pump from Waters Assoc. (Millford, MA, U.S.A.) was connected to a Rheodyne (Berkeley, CA, U.S.A.) 7125 injector. The injection volume (loop) was 50 μl. The column (25 cm × 7 mm I.D.) was obtained from Merck (Darmstadt, F.R.G.), filled with LiChrospher Diol Si 200 (pore diameter 200 Å, bead diameter 10 μm). This support was chosen because of the weak adsorption of haemoglobin on it.

A Pye Unicam (Cambridge, U.K.) Type LC3 detector was used, monitoring the solutions at 415 nm. The column was maintained at 21.5°C by circulating water.

All measurements were performed with Hb solution in 50 m*M* phosphate buffer (pH 7.4), except for Hb-CO, for which 50 m*M* Tris buffer (pH 7.4) was used.

Flash photolysis

The measurements were made with a Durrum-Gilson stopped-flow apparatus, interfaced with a Data General microcomputer (Nova 2/16K), programmed for the acquisition and treatment of data. A Phase R dye laser with Rhodamine 6G was used. Carboxyhaemoglobin solutions were $1.24 \cdot 10^{-5}$ *M* (tetramer) in Tris buffer (50 m*M*) pH 7.4.

Proteins

All proteins studied were obtained from Sigma (St. Louis, MO, U.S.A.), except carbonic anhydrase, purchased from Boehringer (Mannheim, F.R.G.). Dextrans were obtained from Sigma or Pharmacia (Uppsala, Sweden) (M_w = 70000 and 10000). Haemoglobin A and S were prepared from freshly drawn red blood cells, collected in NaCl, EDTA and glucose media. The red blood cells were washed three times with isotonic saline, lysed in water and centrifuged in order to remove cell membranes.

The reactivity of the cysteine β93 residue of human haemoglobin was used to modify Hb with the thiol reagents dithiodiformamidine, iodoacetamide or oxidized glutathione by the following procedure[14]. Haemoglobin was incubated in 0.15 *M* phosphate buffer (pH 7.4) at 37°C in a water-bath shaker for 90 min with dithiodiformamidine (DFA) or iodoacetamide with a molar ratio r = [reagent]/[Hb] = 20. After the reaction, the solution was extensively dialysed in the final buffer.

The modification of Hb by oxidized glutathione (GLU) has previously been described by Beuzard *et al.*[15]. The extent of the reaction can be assessed by isoelectric focussing on polyacrylamide slabs[14]. The percentage of modified Hb was found to be 100% for iodoacetamide, but 75% for both DFA and GLU. Therefore, it was necessary to separate the unmodified Hb by preparative chromatography on DEAE-cellulose. The sample was first dialysed against 0.2 *M* glycine buffer (pH 7.8) and then applied to a column of DEAE-cellulose, equilibrated with the same buffer. The modified haemoglobin was eluted by steps of increasing NaCl concentration of 0.025 then 0.05 *M*.

Carboxyhaemoglobin was obtained by bubbling an excess of CO into the Hb solution.

RESULTS

Fig. 1 represents the calibration graph $-\log M = f(\sigma)$, where M is the molecular weight of the sample. In the protein group, despite some dispersion of the points due to adsorption or ionic effects, the calibration graph can be drawn between $V_0 = 5.2$ ml and $V_0 + V_p = 10.2$ ml (^2H_2O). The calibration graph obtained with dextran samples confirms these values. We injected concentrated ($5 \cdot 10^{-3}$ M) and dilute ($5 \cdot 10^{-8}$ M) $HbA(O_2)$ solutions. The retention volumes correspond to molecular weights very close to the values of Hb tetramer and dimer, respectively (Fig. 1). These last values were confirmed by the use of the saturation method described below. These results indicate that the chromatography Hb on the column is free from adsorption phenomena. We have assumed that it is also the same for all the other Hb species studied, and that the calibration curve $-\log M = f(\sigma)$ is linear in the domain of interest.

Determination of $HbA(O_2)$ dissociation constant

We applied the saturation method to a series of $HbA(O_2)$ solutions at various concentrations. The Hb retention was obtained by injection of small volume (50 μl) of solvent, which gives negative peaks (Fig. 2), the elution volumes of which are measured. The protein retention volumes are plotted in Fig. 3 as a function of $-\log[Hb]$. It appears from the results that for concentrated solutions the retention volume reaches a limiting volume $V_T = 7.03$ ml, corresponding to the Hb tetramer, and for the dilute solutions a limiting volume of $V_D = 7.87$ ml (Hb dimer).

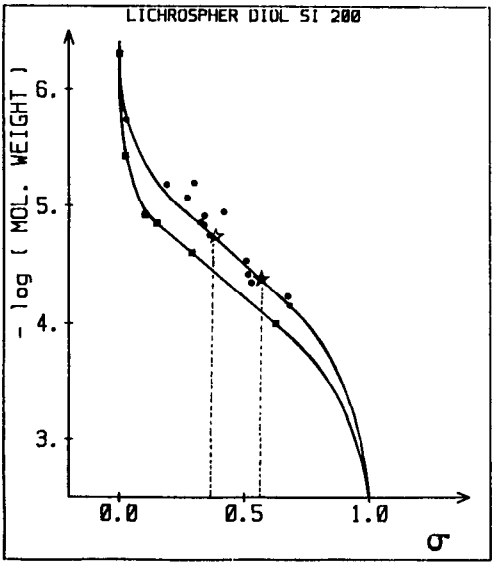


Fig. 1. Calibration graph for LiChrospher Diol Si 200 column. ■, Dextrans; ●, proteins. Thyroglobulin, M_w 660 000; ferritin, 440 000; aldolase, 160 000; γ-globulins, 153 000; alkaline phosphatase, 116 000; conalbumin, 86 200; transferrin, 77 000; human serum albumin, 69 000; bovine serum albumin, 68 000; catalase, 58 000; ovalbumin, 44 000; pepsin, 34 000; carbonic anhydrase, 31 000; myoglobin, 17 200; ribonuclease, 13 700. ☆, $HbA(O_2)$, $5 \cdot 10^{-3}$ M; ★, $HbA(O_2)$, $5 \cdot 10^{-8}$ M.

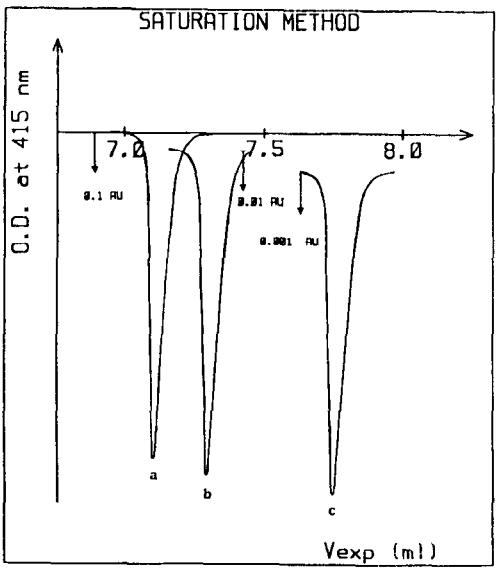


Fig. 2. Saturation method. Injection of 50 μl of 0.05 M aqueous phosphate buffer (pH 7.4). Eluent: (a) 10^{-5} M HbA(O_2), detector sensitivity s = 1; (b) 10^{-6} M HbA(O_2), s = 0.1; (c) 10^{-7} M HbA(O_2), s = 0.01.

From these values, we calculated the equilibrium dissociation coefficient, α, for each solution from the retention volume V by eqn. 15.

Fig. 3 represents the change of α with $-\log[\mathrm{Hb}]$. As

$$^4K_2 = \frac{4[\mathrm{Hb}]\alpha^2}{1 - \alpha}$$

it is relatively easy to obtain $^4K_2$ with several points. The accuracy is better when α is far from 0 or 1, and we calculated $^4K_2$ with a value of α = 0.39, where $^4K_2$ = [Hb]. In this way we obtained the value $^4K_2 = (1.3 \pm 0.5) \cdot 10^{-6}$ M, which can be compared with the value obtained by Brumbaugh and Ackers[9] of $^4K_2 = (1.1 \pm 0.5) \cdot 10^{-6}$ M. This good agreement demonstrates the reliability of the technique.

Zonal chromatography

In order to determine the elution factor, f, defined above, we injected small samples (50 μl) of Hb(O_2) solutions of different concentrations (C_0) and measured their retention volumes after elution with buffered water. The results are reported in Figs. 4 and 5. The equivalent dissociation coefficient, α_{eq}, after elution was calculated from the retention volume using eqn. 15 and plotted as a function of $-\log C_0$ (Fig. 6).

After elution from the column, the Hb sample is, in its dissociated state, equivalent to a solution of concentration C_{eq} saturating the column. We calculated the elution factor, f, from eqn. 18, where α_0 can be obtained graphically from Fig. 3 or 6. Thus, for each value of C_0 injected we have the values of α_0 (before elution) and α_{eq} (after elution). In Fig. 7 f is plotted as a function of α_{eq}. This factor, which is

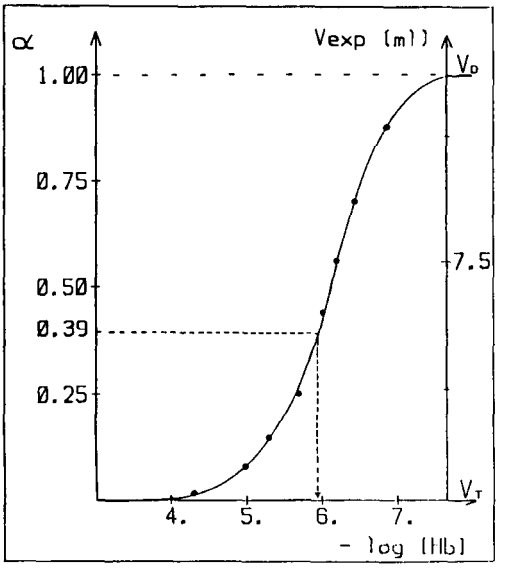


Fig. 3. Dissociation coefficient, α, and $HbA(O_2)$ retention volume for solutions of different concentrations, measured by the saturation method.

independent of $^4K_2$, permits C_0/C_{eq} to be obtained for a given α_{eq} with different haemoglobins.

Study of different haemoglobins

We studied HbS and chemically modified Hb on the cysteine β93 thiol func-

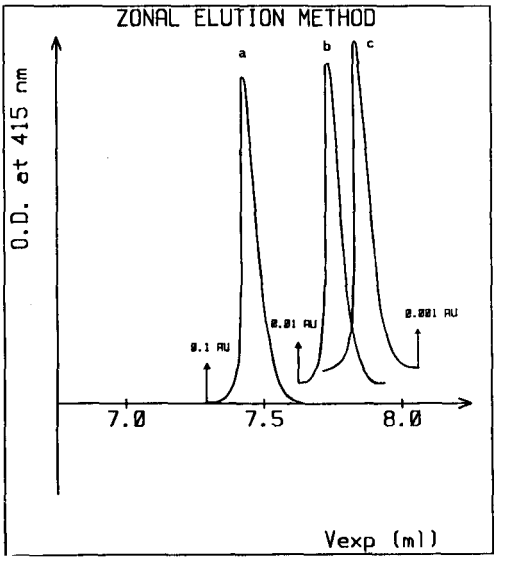
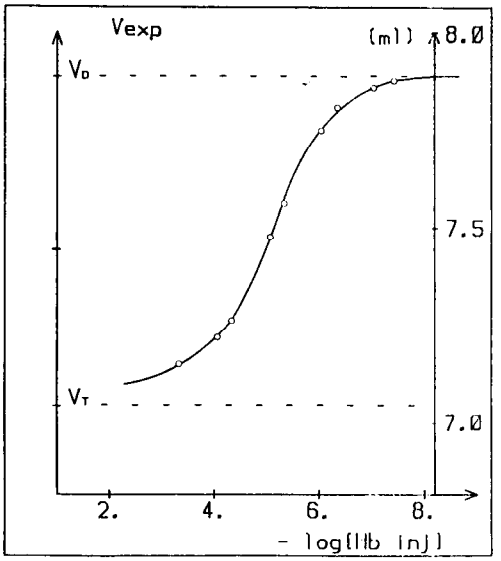


Fig. 4. Zonal elution method: injection of 50 μl of $HbA(O_2)$ at concentration C_0 in phosphate buffer. (a) $C_0 = 10^{-5}$ *M*, detector sensitivity $s = 1$; (b) $C_0 = 10^{-6}$ *M*, $s = 0.1$; (c) $C_0 = 10^{-7}$ *M*, $s = 0.01$. Eluent, 0.05 *M* phosphate buffer (pH 7.4).



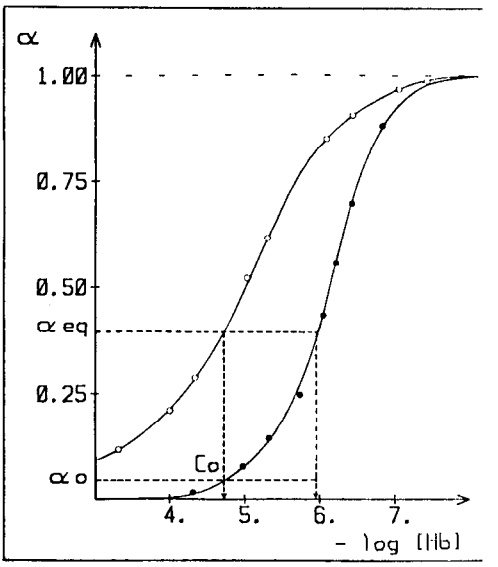


Fig. 5. Hb retention volume of solutions of different concentrations, obtained, by the zonal elution method, as a function of Hb concentration.

Fig. 6. Dissociation coefficient of $HbA(O_2)$ after chromatography on Lichrospher Diol Si 200. ○, Elution method; ●, saturation method.

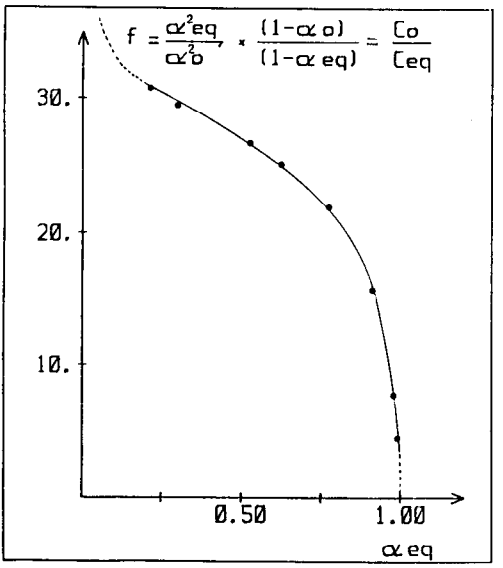
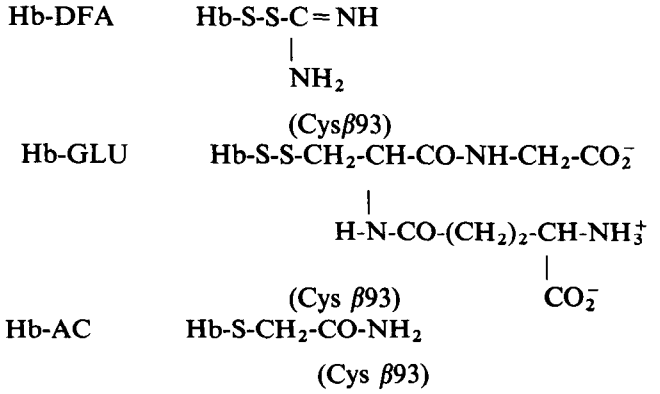


Fig. 7. Elution factor as a function of dissociation coefficient, α_{eq} (after chromatography).

tions. In these instances, the residues added to the SH groups were formamidine, glutathione and acetamide, as shown below.



In Fig. 8, α_{eq}, obtained from measurements of the retention volume of each protein, is plotted against $-\log[Hb]$. The elution factor in Fig. 7 gives C_0/C_{eq} for each value of α_{eq}. As the injected sample concentration, C_0, is known, we obtain the corresponding $C_{eq} = C_0/f$. Hence, it is possible to express α_{eq} as a function of C_{eq} (Fig. 9). All the curves for the different Hbs have similar shapes and are parallel to the curve of $HbA(CO_2)$ previously obtained by the saturation method. The values of ${}^4K_2$ for each haemoglobin were determined at $\alpha_{eq} = 0.39$ and are reported in Table I.

In order to compare this chromatographic method with the results of flash photolysis experiments, we measured the dissociation constants of HbA(CO), HbS(CO), HbA(CO)-DFA and HbA(CO)-GLU and compared them with the values obtained by flash photolysis. The results are presented in Table II. There is a very good agreement between the two techniques.

TABLE I

Hb TETRAMER–DIMER EQUILIBRIUM

Dissociation constants and free enthalpy. Conditions: phosphate buffer, 0.05 *M*; pH 7.4; temperature 21.5°C.

Haemoglobins	${}^4K_2$ (· 10^{-6} *M*)	${}^4\Delta G_2$ *(kcal)*
$HbA(O_2)$	1.3 ± 0.5	−7.9 ± 0.2
HbA(CO)	2.1 ± 0.5	−7.6 ± 0.2
$HbS(O_2)$	1.2 ± 0.5	−8.0 ± 0.2
HbS(CO)	1.1 ± 0.5	−8.0 ± 0.2
$HbA(O_2)$-AC	20 ± 5	−6.3 ± 0.2
$HbA(O_2)$-DFA	14 ± 5	−6.5 ± 0.2
HbA(CO)-DFA	13 ± 5	−6.6 ± 0.2
$HbA(O_2)$-GLU	9.3 ± 0.8	−6.8 ± 0.2
HbA(CO)-GLU	13 ± 5	−6.8 ± 0.2

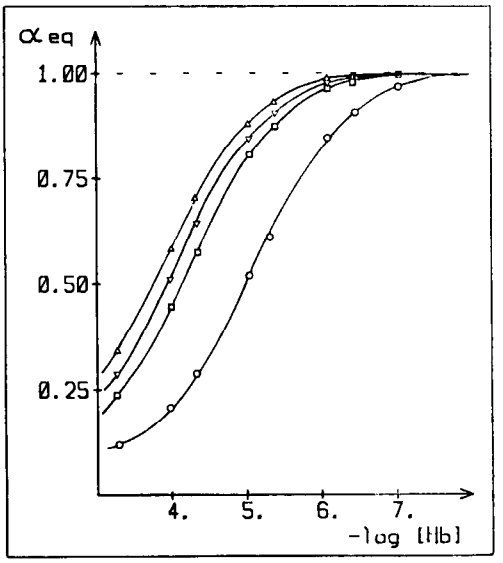


Fig. 8. Dissociation coefficients of different Hbs, measured by the elution method, as a function of injected Hb concentration. ○, $HbA(O_2)$; □, $HbA(O_2)$-GLU; ▽, $HbA(O_2)$-DFA; △, $HbA(O_2)$-AC.

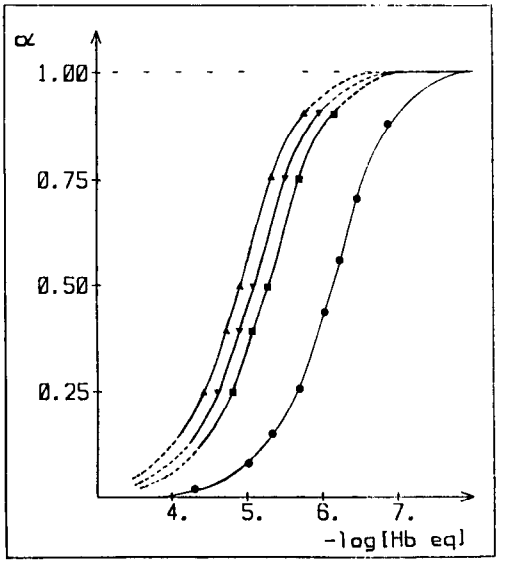


Fig. 9. Dissociation coefficients of different Hbs, measured by the elution method, as a function of equivalent equilibrium concentration, ●, $HbA(O_2)$; ■, $HbA(O_2)$-GLU; ▼, $HbA(O_2)$-DFA; ▲, $HbA(O_2)$-AC.

TABLE II

DISSOCIATION CONSTANTS OF Hb TETRAMER–DIMER EQUILIBRIUM

Conditions: Tris–HCl, 0.05 *M*; pH 7.4; temperature, 21.5°C.

Haemoglobins	$^4K_2$ ($\cdot 10^{-6}$ *M*)	
HbA(CO)	4.1 ± 0.5	Flash photolysis
	6.3 ± 0.5	Elution chromatography
HbS(CO)	7.4 ± 0.5	Flash photolysis
	5.4 ± 0.5	Elution chromatography
HbA(CO)-DFA	45 ± 5	Flash photolysis
	51 ± 5	Elution chromatography
HbA(CO)-GLU	48 ± 5	Flash photolysis
	52 ± 5	Elution chromatography

DISCUSSION

The zonal chromatographic method depends on the experimental evaluation of an elution factor that is independent of the protein being tested. This point is currently under further investigation. In this respect, possible kinetic effects must be considered. In both of the methods described here, we noticed neither appreciable asymmetry nor an increase in the Hb peak width under different elution conditions. The possible variation of the Stokes radius of Hb tetramer or dimer after chemical modification could also be questioned. Using high dilution conditions we observed the same limiting value of the retention volume, corresponding to the dimer retention, V_D, for both modified and normal Hb. On the other hand, recent results obtained in our laboratory[16] by high-resolution NMR spectroscopy have shown that the perturbation induced by thiol reagents bound to cysteine β93 of Hb is localized, and does not affect the overall conformation of the protein. The interaction of modified Hb with the stationary phase must also be a cause of retention variations, but the invariability of the different Hb retentions at high dilution makes this possibility unlikely. With reversible binding of several drugs on human serum albumin we have never observed any variation of the protein retention on LiChrosorb Diol columns[17], and the same applies to haemoglobin.

CONCLUSION

The saturation method in high-performance size-exclusion chromatography was used to determine the dissociation constant of the tetramer–dimer equilibrium of HbA(CO). The method is rapid, but requires a large amount of material. In order to obtain valid measurements with only minute amounts of protein, zonal chromatography was used for the characterization of discussion equilibria. The results of measurements of dissociation constants of different haemoglobins by this technique and by the flash photolysis method are in good agreement, thereby validating the present chromatographic method. From these results, it appears that thiol reagents covalently attached to Hb alter the association constant of the tetramer–dimer equilibrium by one order of magnitude.

ACKNOWLEDGEMENT

The authors are very grateful to Dr. S. Edelstein for helpful discussions.

REFERENCES

1 G. A. Gilbert, *Discuss. Far. Soc.*, No. 20 (1955) 68.
2 G. A. Gilbert, *Proc. R. Soc. London, Ser. A*, 250 (1959) 377.
3 G. A. Gilbert, *Nature (London)*, 212 (1966) 296.
4 G. A. Gilbert, *Anal. Chim. Acta*, 38 (1967) 275.
5 D. J. Windsor and H. A. Sheraga, *Biochemistry*, 2 (1963) 1263.
6 D. J. Windsor and H. A. Sheraga, *J. Phys. Chem.*, 68 (1964) 338.
7 G. A. Gilbert and G. L. Kellett, *Fed. Eur. Biochem. Symp.*, 6 (1968) 73.
8 L. W. Nichol, A. G. Ogstom and D. J. Windsor, *Arch. Biochem. Biophys.*, 121 (1967) 517.
9 E. E. Brumbauch and G. K. Ackers, *J. Biol. Chem.*, 243 (1968) 6315.
10 G. K. Ackers, E. E. Brumbaugh, S. Ip and H. R. Halvorson, *Biophys. Chem.*, 4 (1976) 171.
11 R. Valdes, L. P. Vickers, H. R. Halvorson and G. K. Ackers, *Proc. Nat. Acad. Sci. U.S.*, 75 (1978) 5493.
12 B. Sebille, N. Thuaud and J. P. Tillement, *J. Chromatogr.*, 180 (1979) 103.
13 S. J. Edelstein, M. J. Rehmar, J. S. Olson and Q. H. Gibson, *J. Biol. Chem.*, 245 (1970) 4372.
14 M. C. Garel, Y. Beuzard, J. Thillet, C. Domenget, J. Martin, F. Galacteros and J. Rosa, *Eur. J. Biochem.*, 123 (1982) 513.
15 Y. Beuzard, M. C. Garel, J. Caburi-Martin, C. Domenget, F. Galacteros and J. Rosa, *Blood*, 62 (1983) 530.
16 C. T. Craescu, J. Mispelter, C. Schaeffer and Y. Beuzard, *J. Biol. Chem.*, (1985) submitted for publication.
17 B. Sebille, N. Thuaud and J. P. Tillement, *J. Chromatogr.*, 167 (1978) 159.