Journal of Chromatography, 395 (1987) 511–521
Elsevier Science Publishers B.V., Amsterdam — Printed in The Netherlands

CHROMSYMP. 1228

ZONAL HIGH-PERFORMANCE AFFINITY CHROMATOGRAPHY AS A TOOL FOR PROTEIN INTERACTION STUDIES WITH SPECIAL REFERENCE TO THE LIPASE–COLIPASE COMPLEX

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SUMMARY

The technique of zonal high-performance affinity chromatography applied to the lipase–colipase system (lipase B as eluted acceptor and colipase as silica-bonded ligand) gave qualitatively the same results as conventional affinity chromatography. The elution volume of the acceptor increases with decreasing load introduced at constant volume into the column of ligand-bonded silica. This led to the use of a mathematical treatment for calculating the dissociation constant ($\bar{K}_D$) of the lipase–colipase complex. The influence of some physical and chemical chromatographic parameters was studied. Increasing temperature and flow-rate reduced the affinity of lipase for colipase, whereas it was only slightly modified by increasing the ionic strength. The $\bar{K}_D$ value was minimal and equal to $0.1 \cdot 10^{-6}$ *M* at pH 4.7 and $0.38 \cdot 10^{-6}$ *M* at pH 6.5, after correction for the flow-rate. The latter value is similar to that obtained by more conventional techniques. The absence of some marked $\bar{K}_D$ modifications by ionic strength and the value of ΔS for the complex association obtained by temperature studies suggest the intervention of mixed hydrophobic–ionic interactions in the formation of the lipase–colipase complex. Their respective importances are discussed.

INTRODUCTION

High-performance affinity chromatography (HPAC) can be used for studying macromolecular interactions such as antigen–antibody[1–3], enzyme–substrate[4,5], concanavalin–sugar[6,7] and hormonal peptide–protein[8,9]. We recently described a conventional affinity chromatographic (CAC) technique[10], employing a colipase-bonded acrylamide–agarose gel, with the purpose of calculation the dissociation constant, $\bar{K}_D$, between the eluted lipase and the immobilized colipase by means of a mathematical treatment. A zonal procedure was chosen for the limited amounts of disposable, purified material. Transposition of CAC to HPAC is proposed here as a rapid means of describing lipase-B–colipase interactions (a model of protein interactions). The influence of some physical (temperature, flow-rate) and chemical (pH, ionic strength) chromatographic parameters on the protein–protein association is discussed.

0021-9673/87/$03.50

EXPERIMENTAL

Preparation of lipase–colipase and enzyme activity

Porcine pancreatic lipase and colipase were purified according to Rovery *et al.*[11] and Léger *et al.*[12], respectively. The purity was verified by polyacrylamide gel electrophoresis. Lipase activities were measured potentiometrically at 20°C and pH 8.7 with a Metrohm (Herisau, Switzerland) apparatus, including an E 510 pH meter, an E 473 Impulsomat and a E 415 Multi-dosimat, on 0.5 ml of an emulsion containing 250 m*M* tributyrin (Prolabo, Paris, France) as a substrate and 2.5 m*M* sodium taurodeoxycholate (Sigma, St. Louis, MO, U.S.A.). The complete incubation medium also contained 9.5 ml of 10 m*M* Tris–HCl buffer, 150 m*M* sodium chloride, 16 m*M* calcium chloride and an excess of colipase.

Preparation of the chromatographic system

Activation of the coated porous silica. LiChrosorb-Diol (Merck, Darmstadt, F.R.G.) is a glycidoxypropyltrimethoxysilane-coated 10-μm silica with a mean pore diameter of 10 nm and a separation range from 10 to 100 kD for globular proteins. It was suspended in 0.1 *M* periodic acid (1.1 g for 150 ml) for oxidation of the diol to aldehyde functions. After stirring for 1 h at 20°C, the silica was washed with distilled water and equilibrated in a 0.1 *M* (pH 7.4) sodium phosphate buffer.

Colipase bonding on the activated coated silica. The following procedure is similar to that described previously[12]. The activated coated silica was suspended in the phosphate buffer (3 ml for 30 mg of colipase), stirred for 16 h at 4°C and washed with 30 ml of 0.5 *M* potassium phosphate (pH 7.6)–0.15 *M* sodium chloride. An amount of 8.15 mg of colipase was bonded. The same procedure was repeated, leading to a final bonded amount of 16.3 mg. The colipase in excess that was not bound to the activated support was assayed in the washing buffer by measuring the absorbance at 280 nm using an extinction coefficient $E_1^{1\%}{}_{cm}$ of 4.0 and by the protein assay according to the Lowry procedure. The unreacted aldehyde groups were blocked as follows: the treated silica was mixed for 16 h at 4°C in the presence of 0.5 *M* ethanolamine–potassium phosphate buffer and washed with 200 ml of distilled water, then with 100 ml of ethanol, and dried under vacuum.

Column preparation. A 5 cm × 0.46 cm I.D. column was filled with a suspension of the colipase-bonded silica in 10 ml of carbon tetrachloride under a pressure of 300 bar according to the procedure in ref. 13. The column was then washed successively with 50 ml of methanol, 50 ml of distilled water and 50 ml of elution buffer. It contained 420 mg of silica and 6.2 mg of bonded colipase (15 mg colipase per gram of silica), corresponding to a total volume of 0.83 ml and to a concentration of the total bonded colipase in the column of $[\bar{C}]_t = 7.45 \cdot 10^{-4}$ *M*.

Instrumentation

The experiments were conducted with HPLC apparatus from Beckman (Gagny, France) with a Beckman Model 112 solvent-delivery system, an Altex injector fitted with a 50-μl sample injection loop and a Beckman Model 160 UV spectrophotometric detector, fitted with a 254-nm filter (this wavelength increases the detection limit of lipase with this type of detector, a correction factor of 1.85 yielding the absorbance at 280 nm and the lipase concentration by using an $E_1^{1\%}{}_{cm}$ of 13.0). Chromatographic profiles were typically recorded at 0.005 a.u.f.s.

Calculation of $\bar{K}_D$

The mathematical treatment for the calculation of $\bar{K}_D$ has been described previously[10]. Briefly, the basic simplified expression obtained is

$$\frac{1}{K_p} = \frac{1}{[\bar{C}]_a}[L] + \frac{\bar{K}_D}{[\bar{C}]_a} \quad (1)$$

where K_p, the partition coefficient, depends only on the interactions between eluted lipase and bonded colipase. Its expression is $(V_e - V'_e)/(V_T - V_0)$, where V_e is the elution volume of lipase in the presence of interacting bonded colipase, V'_e the elution volume of lipase in the absence of colipase interactions (assessed by means of microbial lipases of *Rhizopus arrhizus* and *Geotrichum candidum*, with a molecular weight close to that of the pancreatic lipase and which does not interact with colipase[14]), V_T the total volume occupied by the liquid phase in the column and V_0 the void volume determined using Blue Dextran. In this instance, V_0 = 0.42 ml and V_T = V'_e = 0.63 ml (this experimental value is confirmed by the theoretical value). The meanings of the other symbols in eqn. 1 are $\bar{K}_D$ = dissociation constant of the lipase–bonded colipase complex, $[\bar{C}]_a$ = concentration of colipase actually active for engaging in some interactions with the lipase present in the mobile phase and $[L]$ = lipase "operational" concentration in the column, which can be deduced from the concentration of lipase at the summit of the peak leaving the column (see ref. 10). Plotting eqn. 1 leads to the calculation of $[\bar{C}]_a$ and $\bar{K}_D$ (see Fig. 2).

RESULTS

Fig. 1 illustrates the chromatographic elution profiles of lipase B for several amounts loaded on to the column of colipase-bonded silica with a constant injection volume of 50 μl and at pH 6.5. The elution volume increased with decreasing load of lipase. The partition coefficient increased by a factor of 1.6 when the amount loaded was reduced by 80%. The plot of eqn. 1 is shown in Fig. 2. Its linearity was confirmed, leading to the values $0.68 \cdot 10^{-6}$ *M* for $\bar{K}_D$ and $8.7 \cdot 10^{-6}$ *M* for $[\bar{C}]_a$. A $[\bar{C}]_a/[\bar{C}]_t$ ratio of 1.1% was found.

Fig. 3A shows the increasing retention of lipase with decreasing pH (ionic strength values between 0.1 and 0.18). The partition coefficient decreased from 30 to 5.3 when the pH increased from 4.7 to 7.2. The elution volumes showed a maximal value at pH 4.7 (Fig. 3A), corresponding to a minimal $\bar{K}_D$ of $0.22 \cdot 10^{-6}$ *M* (Fig. 3B).

As shown in Fig. 4A, the partition coefficient decreased on increasing the temperature. $\bar{K}_D$ was reduced by a factor of 2.3 on going from 4 to 28°C. This strongly suggests a higher stability of the complex at low temperatures (Fig. 4B and C).

Increasing concentration of sodium chloride in the elution buffer at pH 6.5 led to a small decrease in the elution volume and therefore in the partition coefficient (Fig. 5A). The $\bar{K}_D$ values were not greatly affected* (Fig. 5B).

* The slight differences between the values in Figs. 1 and 5 were due to the time elapsed between the experiments, leading to weak denaturation of the bonded colipase, without affecting the significance of the observed process.

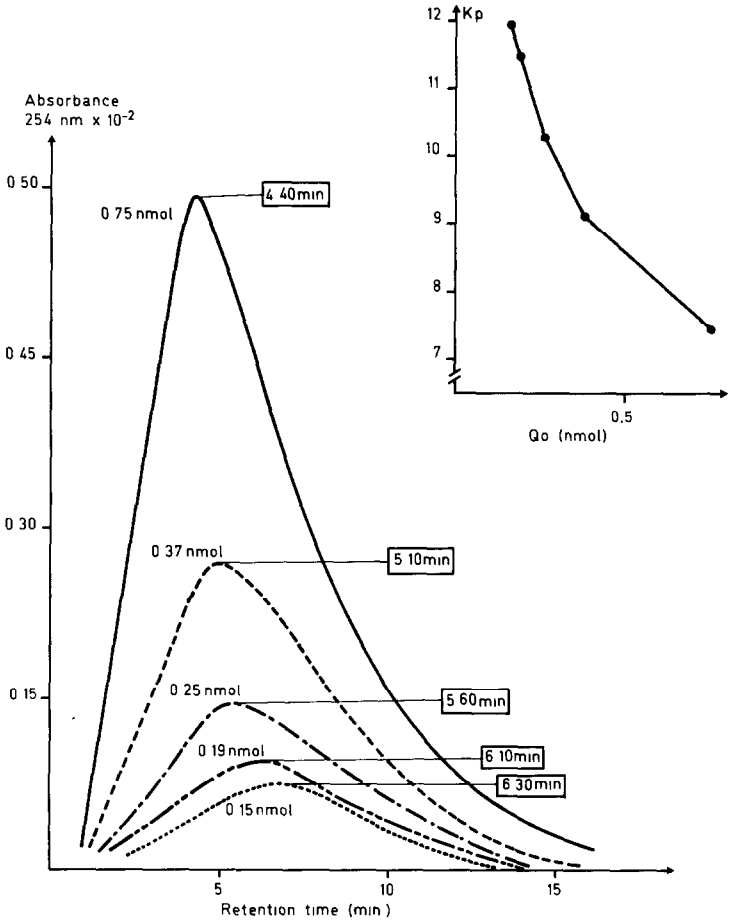

Fig. 1. Chromatographic profiles of lipase B for different loads, Q_0, on the column (5 × 0.46 cm I.D.) of colipase-bonded LiChrosorb-Diol. Eluting buffer, 4.2 m*M* Na_2HPO_4–7.4 m*M* NaH_2PO_4–80 m*M* NaCl (pH 6.5; I = 0.1); injection volume, 50 μl; flow-rate, 0.5 ml/min; temperature, ambient.

A decrease in flow-rate (Fig. 6A and B) from 0.6 to 0.05 ml/min resulted in (i) an increase in the elution volume of lipase in the range 0.6–0.1 ml/min (*i.e.*, 0.08–0.012 cm/s linear velocity), equivalent to an increase in K_p or a decrease in $\bar{K}_D$, and (ii) a steady state for values equal to or lower than 0.1 ml/min (*i.e.*, 0.012 cm/s). The size of the variations was much more marked at the low lipase load.

DISCUSSION

The value of $\bar{K}_D$

The $\bar{K}_D$ value was not very different from those obtained by other techniques under similar chemical conditions: (i) by calorimetry[15], $0.5 \cdot 10^{-6}$ *M*; (ii) by the two-phase partition technique[16], $0.3 \cdot 10^{-6}$ *M*; (iii) by CAC[10,17], $1.0 \cdot 10^{-6}$ and $1.9 \cdot 10^{-6}$ *M*. The results presented in this paper show that use of HPAC with the

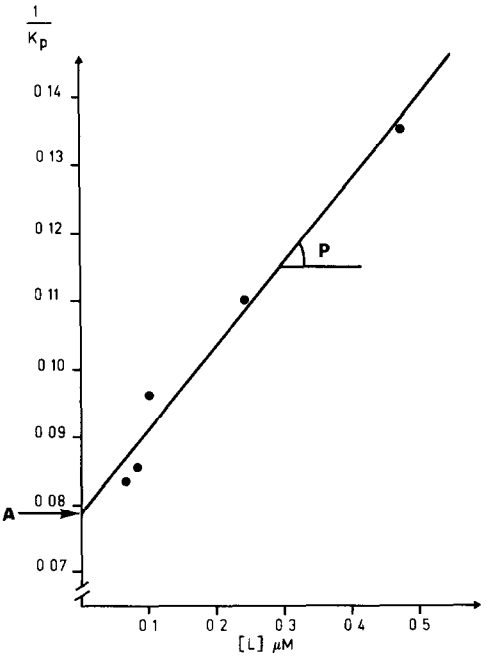

Fig. 2. Dependence of the reciprocal of the partition coefficient on the "operational" concentration of lipase within the column during elution (see text and ref. 10). $1/K_p = (1/[\bar{C}]_a) \cdot [L] + \bar{K}_D/[\bar{C}]_a$. $K_D = A/P = 0.68 \cdot 10^{-6}$ M; $[\bar{C}]_a = 1/P = 8.66 \cdot 10^{-6}$ M; $[\bar{C}]_a/[\bar{C}]_t = 1.15\%$. $A = 0.079$; $P = 0.115 \cdot 10^6$ M^{-1}. A = intersection of the straight line with the y-axis; P = slope of the plot.

previously proposed mathematical treatment[10] represents a reliable and very promising means of calculating dissociation constants rapidly. The bonding of colipase on LiChrosorb-Diol seems not to affect the lipase–colipase interactions. It should be noted that the value of $\bar{K}_D$ relating to the unbonded colipase could be determined by adding increasing amounts of free colipase to the elution buffer and the complete expression of eqn. 1:

$$\frac{1}{K_p} = \frac{1}{[\bar{C}]_a}\left(1 + \frac{[C]}{K_D}\right)[L] + \left(1 + \frac{[C]}{K_D}\right)\frac{\bar{K}_D}{[\bar{C}]_a} \qquad (2)$$

where $[C]$ = the concentration of colipase in the buffer and K_D = dissociation constant of lipase–unbonded colipase. Eqn. 2 is preferably transformed into

$$\frac{p}{p_0} = \frac{1}{K_D}[C] + 1$$

where p and p_0 are the slopes of the plot shown in Fig. 2 in the presence and absence of free colipase in the buffer, respectively.

The bonded colipase assumed to be active, *i.e.*, actually capable of interacting

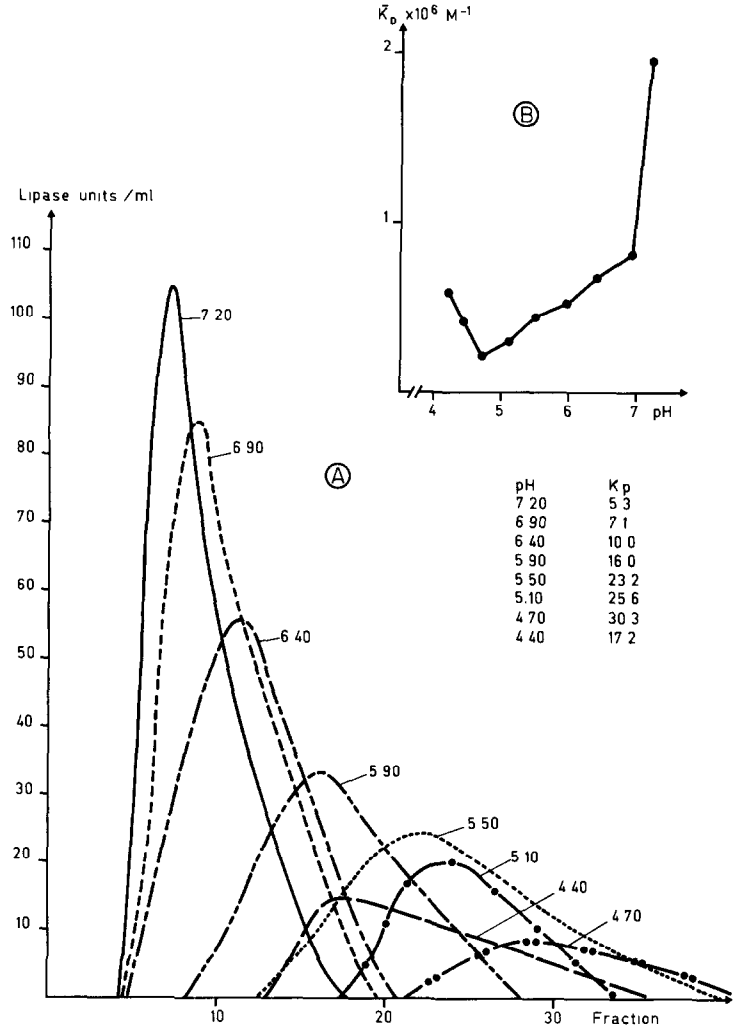

Fig. 3. (A) Chromatographic profiles of lipase B on the same column as in Fig. 1 for different pH values of buffers with ionic strengths from 0.1 to 0.18. Buffers contained 80 m*M* NaCl and CH_3COOH/CH_3COONa or Na_2HPO_4/NaH_2PO_4 for the pH range 4.4–5.5 or 5.9–7.2, respectively. General conditions of chromatography, temperature and flow-rate, similar to those in Fig. 1; load on the column, Q_0, 0.75 nmol. (B) Dependence of $\bar{K}_D$ on pH.

with lipase, is very low (1.1%), much lower than that obtained in CAC[10]. It has already been reported that the fraction of immobilized ligand accessible to the eluted acceptor seems to be low under these chromatographic conditions[18]. This is of great importance but difficult to explain. On the other hand, starting from the values of the dissociation constant obtained here, similar to those obtained by workers employing other techniques[16,19], it can be concluded that this active fraction is low, essentially owing to steric hindrance of the association, or more precisely to a likely

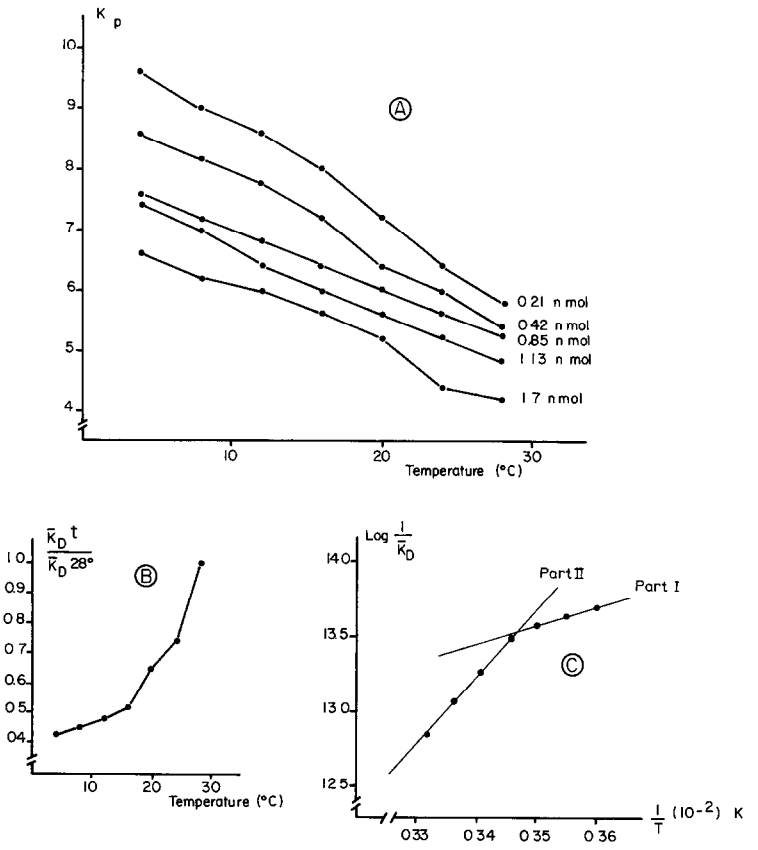

Fig. 4. (A) Plot of partition coefficient *versus* temperature. (B) Relative variations of the dissociation constant with temperature. (C) Van 't Hoff plot of $\bar{K}_D$ with $\Delta H/R$ as the slope of the straight lines: part I $\Delta H_I = -2.4$ kcal/mol, part II $\Delta H_{II} = -8.7$ kcal/mol, leading to $\Delta S_I = +18$ cal/mol/K and $\Delta S_{II} = -3$ cal/mol/K by means of the Gibbs relation.

unsuitable orientation of bonded colipase for the lipase association process, rather to a partial colipase (protein) denaturation.

The most significant point is that assessment of the ratio $[\bar{C}]_a/[\bar{C}]_t$ is essential for the correct evaluation of protein–protein interactions. Fassina *et al.*[8] recently studied a type of protein interaction by affinity chromatography. $\bar{K}_D$ was calculated by extrapolating to zero load and assuming that all of the bonded ligand is active. We calculated $\bar{K}_D$ values from the experimental data reported in this paper by extrapolating at zero load with (i) 100% or (ii) 1.1% of active colipase, the values obtained being: (i) $0.9 \cdot 10^{-4}$ *M* and (ii) $0.7 \cdot 10^{-6}$ *M*. Provided that the active-to-total colipase ratio was taken into account, the value calculated was not very different from that obtained in this work and refs. 16 and 19. This indicates that (i) extrapolating at zero load and evaluating the "operational" concentration of the acceptor proceeding within the column during elution are very similar procedures and (ii) it is essential to use a method involving assessment of the fractional amount of the active bonded

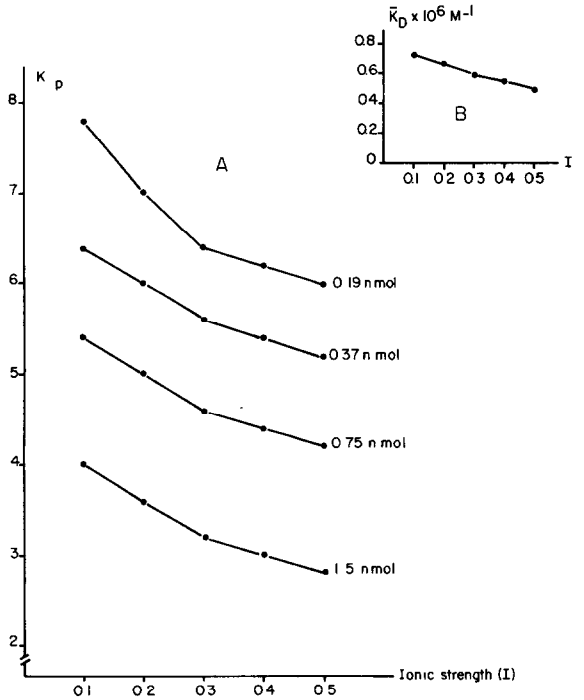

Fig. 5. (A) Plot of partition coefficient *versus* ionic strength at pH 6.5 and ambient temperature. (B) Variation of $\bar{K}_D$ with ionic strength.

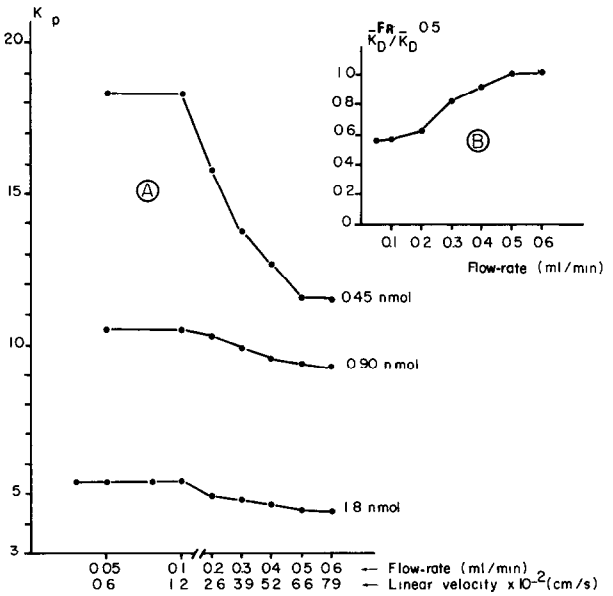

Fig. 6. (A) Plot of partition coefficient *versus* flow-rate or linear velocity at pH 6.5, I = 0.1 and ambient temperature. (B) Relative variations of the dissociation constant, expressed as $\bar{K}_D$ values relative to the value of $\bar{K}_D$ observed at 0.5 ml/min.

ligand in the column. Taking great care that these conditions are met, the determination of $\bar{K}_D$ for a protein–protein complex (or perhaps in instances of interactions between macromolecules) by HPAC is a very rapid, suitable and reliable technique. Taking the effect of flow-rate on $\bar{K}_D$ into account (see below) leads to a further improvement of this procedure.

Effect of flow-rate on $\bar{K}_D$

From a theoretical point of view, it is conceivable that the calculation of an equilibrium constant in HPAC necessarily involves the determination of either the range of flow-rates that really allows the equilibrium conditions to be attained or, conversely, the limit of the linear velocity above which these conditions are not obtained. The present results show that the $\bar{K}_D$ between lipase and colipase decreases with decreasing flow-rate (*i.e.*, the apparent affinity increases) and is stabilized for flow-rates equal to or lower than 0.1 ml/min, at a value probably corresponding to the actual affinity, similar to that in the references already reported. This result is of great importance. It confirms, as expected, the necessity for low linear velocities (below 0.012 cm/s) for reaching the dissociation–association equilibrium in affinity chromatography. However, the meaning of the plateau observed at higher flow-rates (more marked at low loads and obtained very reproducibly) is not clear. Moreover, it could be suggested that there is a discrepancy between this observation and what would be theoretically expected, *i.e.*, a further decrease in K_p. The disagreement could be explained by the involvement of coupled, kinetic and non-linear isotherm effects. It should be noted that the present results clearly show the non-constancy of the partition coefficient in this type of chromatography, contrary to what is frequently assumed in chromatographic processes, but, conversely, the dependence on the concentration of the eluted protein.

From a practical point of view, it is concluded that a correction must be applied to $\bar{K}_D$ for higher flow-rates. It could be conceived that assays are preferably performed at higher flow-rates for convenience, and thereafter a previously assessed correction factor is routinely applied to the value obtained. In the present instance, we found a value of $0.68 \cdot 10^{-6}$ *M* for $\bar{K}_D$ at a flow-rate of 0.5 ml/min. The correction factor is 0.56 to obtain the value of $0.38 \cdot 10^{-6}$ *M* for a flow-rate of 0.1 ml/min.

Effect of pH

The pH of maximal affinity of lipase for colipase found here is in accord with that obtained by two-phase partition[16] or by the CAC technique[10]. At this pH of 4.7 ($I = 0.1$) and after correction for the flow-rate, the value of $\bar{K}_D$ is *ca.* $0.1 \cdot 10^{-6}$ *M*. A maximal density of electric charges could be necessary for obtaining maximal stability of the complex because of the proximity of the pH value to the isoelectric points of both lipase and colipase, *i.e.*, 5.1–5.2 and 5.3–5.5, respectively[20].

Effect of temperature

Fig. 4C shows that the points can be plotted on two lines, indicating that the interactions in the two temperature ranges (above and below 16°C) do not involve the same processes. The values obtained for ΔG, ΔH and ΔS were -7.5 kcal/mol, -2.4 kcal/mol and 18 cal/mol · K, respectively, at 4°C and -7.6 kcal/mol, -8.7 kcal/mol and -3 cal/mol · K, respectively, at 28°C. The positive and negative ΔS at

lower and higher temperatures than 16°C, respectively, suggest different relative importances of hydrophobic and ionic interactions between lipase and colipase in the two temperature ranges.

Effect of ionic strength

Using the technique of two-phase partition, it has previously been found that ionic strength does not greatly affect the lipase–colipase affinity[16]. The results presented here confirm this conclusion at ambient temperature, suggesting not only the occurrence of mixed hydrophobic and ionic interactions, but perhaps also a low predominance of hydrophobic interactions, owing to the uniform effect over the sodium chloride concentration range.

The effects of both temperature and ionic strength on the stability of the lipase–colipase complex are of great importance for a better understanding of the cohesion factors that occur in the association process. Accordingly, the interesting point in this study is to emphasize the role of hydrophobic interactions, which are probably an essential complement for stabilizing ionic interactions between an amine and a carboxyl group (probably aspartate 72, see ref. 10) from lipase and colipase, respectively.

CONCLUSION

The results indicate that HPAC is a very rapid and reliable technique for assessing the affinity characteristics of a protein–protein complex, provided great care is taken to meet certain conditions (*i.e.*, fractional value of the bonded ligand actually active in the column, on the one hand, and either very low linear velocities of the eluent or a correction factor for velocities higher than 0.012 cm/s, on the other). The variations in these characteristics in relation to some physico-chemical variables can be studied. The $\bar{K}_D$ values were $0.38 \cdot 10^{-6}$ *M* at pH 6.5 and $0.1 \cdot 10^{-6}$ *M* at pH 4.7, which is the pH corresponding to the optimal stability of the complex. The value of ΔS at temperatures below 16°C and the absence of a marked effect of ionic strength on the dissociation constant confirm the intervention of mixed hydrophobic and ionic interactions for stabilizing the lipase–colipase complex, and suggest a (slightly) different distribution of each type of interaction in the two temperature ranges (lower and higher than *ca.* 16°C).

REFERENCES

1 C. J. van Oss, R. J. Good and M. K. Chaudhury, *J. Chromatogr.*, 376 (1986) 111.
2 J. R. Sportsman and G. S. Wilson, *Anal. Chem.*, 52 (1980) 2013.
3 J. R. Sportsman, J. D. Liddil and G. S. Wilson, *Anal. Chem.*, 55 (1983) 771.
4 Y. C. Liu and E. Stellwagen, *J. Chromatogr.*, 376 (1986) 149.
5 B. M. Dunn and I. M. Chaiken, *Biochemistry*, 14 (1975) 2343.
6 D. J. Anderson and R. R. Walters, *J. Chromatogr.*, 376 (1986) 69.
7 A. J. Muller and P. W Carr, *J. Chromatogr.*, 357 (1986) 11.
8 G. Fassina, H. E. Swaisgood and I. M. Chaiken, *J. Chromatogr.*, 376 (1986) 87.
9 H. E. Swaisgood and I. M. Chaiken, *J. Chromatogr* , 327 (1985) 193.
10 J.-M. Alessandri, C. Léger and N. Mahé, *Biochimie*, 66 (1984) 663.
11 M. Rovery, M. Boudouard and J. Bianchetta, *Biochim. Biophys. Acta*, 525 (1978) 373.

12 C. Léger, J.-M. Alessandri, G. Kann, M. Charles, T. Corring and J. Flanzy, *Biochim. Biophys. Acta*, 713 (1982) 208.
13 B. Coq, C. Gonnet and J.-L. Rocca, *J. Chromatogr.*, 106 (1975) 249.
14 P. Canioni, R. Julien, J. Rathelot and L. Sarda, *Lipids*, 12 (1977) 393.
15 J. Donner, C. H. Spink, B. Borgström and I. Sjohelm, *Biochemistry*, 15 (1976) 5413.
16 J. S. Patton, C. Albertsson, C. Erlanson and B. Borgström, *J. Biol. Chem.*, 253 (1978) 4195.
17 A Larsson, C. Erlanson and C. Albertsson, *Biochim. Biophys. Acta*, 664 (1981) 538.
18 F. H. Arnold, S. A. Schofield and H. W. Blanch, *J. Chromatogr* , 355 (1986) 1.
19 J. Donner, C. H. Spink, B. Borgström and I. Sjohelm, *Biochemistry*, 15 (1976) 5413.
20 B. Sternby and B. Borgström, *Comp. Biochem. Physiol.*, 68B (1981) 15.