Solution behaviour of *Chromobacter viscosum* and *Pseudomonas* sp. lipases

No evidence of self-association

Neil J. SIMPKIN, Stephen E. HARDING* and Michael P. TOMBS

Department of Applied Biochemistry and Food Science, University of Nottingham, Sutton Bonington, LE12 5RD, U.K.

1. The size of two bacterial lipases was studied by SDS/PAGE, sedimentation velocity and sedimentation equilibrium to test for possible self-association behaviour. 2. M_r values of selected lipases were obtained from SDS/PAGE and sedimentation-velocity measurements, together with an absolute determination by sedimentation equilibrium. 3. The M_r values obtained in a variety of aqueous solvents indicate that lipases do not self-associate in solution, suggesting the absence of surface hydrophobic patches.

INTRODUCTION

Lipases are enzymes of increasing commercial significance, with applications in oil and fat processing and detergents (Macrae & Hammond, 1985). Lipases function interfacially (Semeriva & Dufour, 1972; Brockerhoff & Jensen, 1974), and crude industrial enzymes must compete for the interface with other agents, particularly other proteins. This can lead to reactor inefficiency (Wisdom et al., 1985). A better understanding of this and other significant problems depends on information on the structure and behaviour of lipases in solution and at the interface.

Harding & Tombs (1989) employed incompatible two-phase systems (Albertsson, 1985) to investigate lipase transport in concentrated polymer solutions (as might be found in industrial production situations). The present work has the objective of determining whether or not lipases self-associate in aqueous solution.

Protein–protein association is common, and, since lipases have been thought to have at least one major hydrophobic patch (Roberts & Tombs, 1987), they would be expected to dimerize in solution. When that paper was being prepared, there were no crystal structures available. Since then, high-resolution X-ray crystal structures of *Mucor miehei* lipase (Brady et al., 1990) and human pancreatic lipase (Winkler et al., 1990) have showed the absence of significant surface hydrophobic areas in the crystal. These authors speculate that entry into the interface exposes one. Contact with another lipase molecule might equally do so.

MATERIALS AND METHODS

Chromobacter viscosum and *Pseudomonas* sp. lipases were isolated and purified as described by Roberts & Tombs (1987). By SDS/PAGE they were more than 90 % lipase. Concentrations were determined by weighing dried samples (constant weight over P_2O_5 *in vacuo*). The enzyme activity was unaffected by drying. Guanidine hydrochloride (GuHCl) was also dried. High-speed sedimentation-velocity analysis was in phosphate/chloride buffer, pH 6.8, 0.1 M (4.595 g of $Na_2HPO_4,12H_2O$, 1.561 g of KH_2PO_4 and 2.923 g of NaCl per litre). The same buffer was employed when using low-speed sedimentation equilibrium, with GuHCl, dithiothreitol (DTT) or 1,4-dioxan added where needed.

SDS/PAGE was in a 13 %-acrylamide gel in Tris/glycine buffer, pH 8.3.

Sedimentation-velocity experiments were done in an MSE Centriscan 75 analytical ultracentrifuge with scanning absorption optics, schlieren optics and a monochromator. Concentrations were corrected for radial dilution. For runs employing absorption optics, the rotor speed was 40000 rev./min and the temperature 20 °C. When scanning schlieren optics were used, the rotor speed was 49000 rev./min. Sedimentation coefficients were corrected to standard conditions (water at 20 °C) and extrapolated to zero concentration, according to the equation (see, e.g., Bowen, 1970) (appropriate to globular proteins) $s_{20,w} = s^0_{20,w}(1 - k_s \cdot c)$ where c is the concentration corrected for radial dilution, k_s is a coefficient, $s_{20,w}$ is the sedimentation coefficient corrected to water at 20 °C and $s^0_{20,w}$ is that at 'infinite dilution'.

Sedimentation equilibrium was performed at 23.8 °C in a Beckman model E analytical ultracentrifuge equipped with Rayleigh interference optics and a 5 mW He–Ne laser-light source, by the 'low'- or 'intermediate'-speed method (Creeth & Harding, 1982). Solutions were dialysed to equilibrium against the solvent (Cassassa & Eisenberg, 1964; Creeth & Pain, 1967; Tombs & Peacocke, 1974). Non-ideality effects were minimized by using low loading concentrations (approx. 0.8 mg/ml) and long (30 mm)-path-length cells. 'Whole-cell' weight-average relative molecular masses ($M^0_{r,w}$), were obtained in accordance with Creeth & Harding (1982). The partial specific volume ($\bar{v}$) was calculated from the amino acid compositions of *C. viscosum* lipase (Isobe & Sugiura, 1977) and *Pseudomonas* sp. lipase (Sugiura & Oikawa, 1977) after Cohn & Edsall (1943). This gave 0.73 ml/g for *C. viscosum* lipase and 0.74 ml/g for *Pseudomonas* sp. lipase. We used a $\bar{v}$ value of 0.73 ml/g throughout, for both lipases.

RESULTS AND DISCUSSION

SDS/PAGE

SDS/PAGE analysis of the lipases (Fig. 1) showed that, for both, the mobility of the zone corresponded to a relative molecular mass ($M_{r,SDS}$) of 33000 ($\pm$1500) (Table 1), standard globular proteins being used to calibrate the gel together with a linear least-squares fitting of the calibration plot.

Abbreviations used: GuHCl, guanidine hydrochloride; DTT, dithiothreitol.
* To whom correspondence should be addressed.

Sedimentation analysis

'Whole-cell' weight-average relative molecular masses $(M_{r,w}^0)$ from sedimentation equilibrium and $s_{20,w}^0$ values from sedimentation velocity appear in Table 1. Approximate relative-molecular-mass values $(M_{r,s})$, calculated from $s_{20,w}^0$ (Squire & Himmel, 1979), assuming a spherical shape (a reasonable assumption for lipases; see, e.g. Brady et al., 1990), are also given in Table 1.

Plots of $s_{20,w}$ versus concentration for C. viscosum and Pseudomonas sp. lipases appear in Figs. 2(a) and 2(b), and the coefficients k_s are given in Table 1. Sedimentation profiles for C. viscosum

lipase obtained by using absorption and schlieren optics appear in Figs. 3(a) and 3(b).

With C. viscosum lipase, there is a suggestion of a positive slope at low concentration, which might be due to self-association (cf. Gilbert & Gilbert, 1961). However, since there was no inflexion point in the absorption sedimentation profile (Fig. 3a), with a lipase concentration in the turnover region, self-association was absent. In Fig. 3(b), a small peak sedimenting more slowly than the main peak was resolved some 2 h into the run. This lower-M_r species, not apparently in chemical equilibrium with the main lipase component, could be responsible for the lower sedimentation coefficients at low concentration.

The data in Table 1 support the idea that C. viscosum lipase does not self-associate in solution, since there is no decrease in the apparent $M_{r,w}^0$ in 6 M-GuHCl, 6 M-GuHCl plus 10 mM-DTT or 40% (w/w) 1,4-dioxan, compared with phosphate buffer.
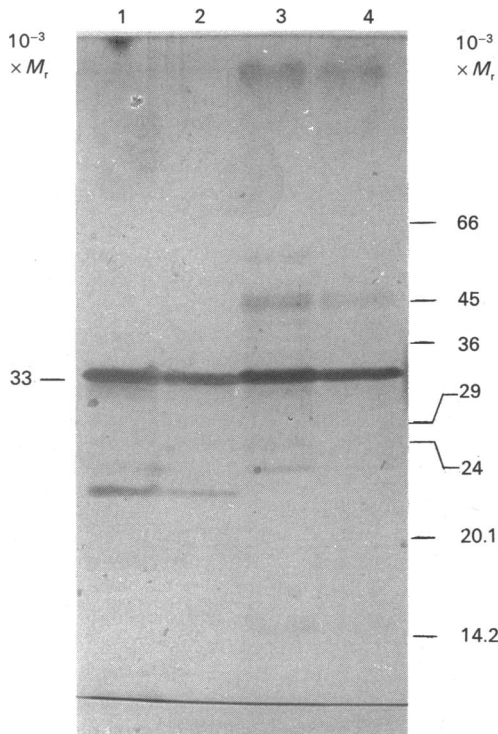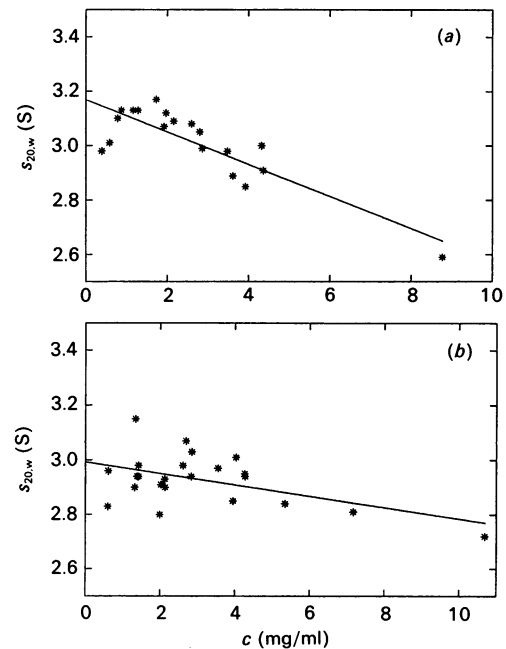

Fig. 1. SDS/PAGE of lipases in Tris/glycine, pH 8.3, in a 13%-acrylamide gel

Calibrating proteins (Sigma MW-SDS-70L; Sigma Chemical Co., Poole, Dorset, U.K.) were in the same gel, but are not shown. Lanes 1 and 2, Pseudomonas sp. lipase; lanes 3 and 4, Chromobacter viscosum lipase.

Fig. 2. Corrected sedimentation coefficient, $s_{20,w}$, plotted versus concentration for (a) C. viscosum lipase and (b) Pseudomonas sp. lipase

The rotor speed used was 40000 rev./min and the temperature 20.0 °C.

Table 1. Ultracentrifugation and SDS/PAGE data for C. viscosum and Pseudomonas sp. lipases

Quoted errors for $M_{r,w}^0$ (weight-average M_r from sedimentation equilibrium) are estimates obtained from graphical extrapolation of a directly determinable point-average molecular mass, M^*, to the cell base (see Creeth & Harding, 1982), together with possible errors in $\bar{v}$ and J_a. Errors quoted for $s_{20,w}^0$ and k_s are standard errors derived from a linear least-squares analysis of the sedimentation data (Fig. 2). The error quoted for $M_{r,SDS}$ (M_r estimated from calibrated SDS/PAGE) is a 5% error estimate, appropriate to gel electrophoresis. The error given for $M_{r,s}$ (the M_r estimated directly from $s_{20,w}^0$) is the standard deviation [see eqn. 8 of Squire & Himmel (1979)]. Abbreviation: PCB, phosphate/chloride buffer.

Measurement	Solvent	C. viscosum lipase	Pseudomonas sp. lipase
$M_{r,w}^0$	PCB	35000 ± 2000	38000 ± 2000
	PCB + 6 M-GuHCl	45500 ± 2500	50000 ± 2500
	PCB + 6 M-GuHCl + 10 mM-DTT	43000 ± 2000	42000 ± 2000
	PCB + 40% 1,4-dioxan	35000 ± 2000	30000 ± 2000
$s_{20,w}^0$(S)	PCB	3.17 ± 0.03	2.99 ± 0.03
k_s(ml/g)	PCB	18.7 ± 0.2	7.0 ± 0.1
$M_{r,s}$	PCB	38700 ± 3500	35400 ± 3200
$M_{r,SDS}$	Tris/glycine	33000 ± 1500	33000 ± 1500

(a)
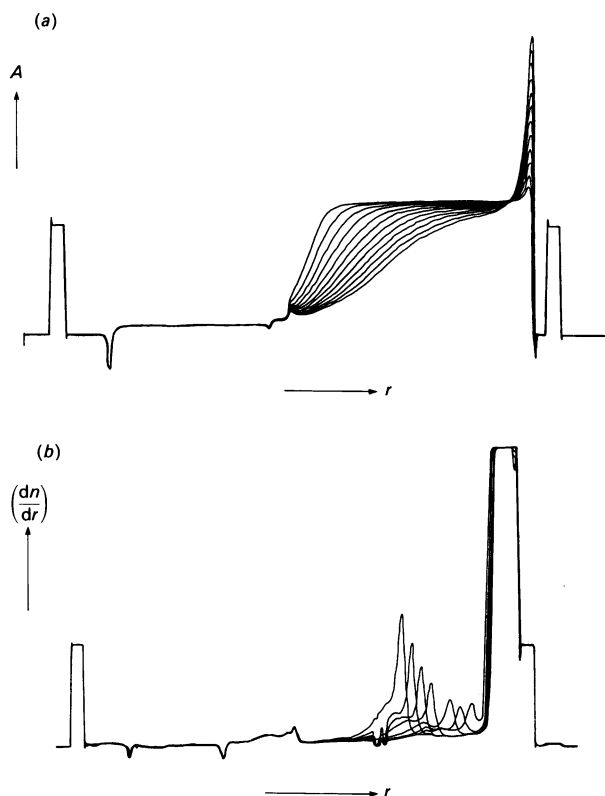
A

(b)

$\left(\frac{dn}{dr}\right)$

Fig. 3. Sedimentation profiles for *C. viscosum* lipase obtained by using (*a*) u.v.-absorption optics and (*b*) schlieren optics

In (*a*) the rotor speed used was 40000 rev./min and the temperature 20.0 °C. In (*b*) the rotor speed was 49000 rev./min and the temperature 20.0 °C.

Also, the values agree with the SDS/PAGE relative molecular mass, $M_{r,SDS}$. *Pseudomonas* sp. lipase yielded similar data (Table 1) and also showed no signs of self-association.

The major source of error in sedimentation equilibrium is in estimating the meniscus solute concentration, J_a ($\pm 10\%$ at best) (Creeth & Harding, 1982). Calculation of partial specific volumes, $\bar{v}$, in 6 M-GuHCl (Lee & Timasheff, 1974) showed that the error in $\bar{v}$ was about 1 %. Thus the M_r values of *C. viscosum* and *Pseudomonas* sp. lipases in phosphate buffer and 40 % 1,4-dioxan are the same, within experimental error.

One anomalous pair of observations that we cannot explain are the $M_{r,w}^0$ values of *C. viscosum* and *Pseudomonas* sp. lipases in the presence of 6 M-GuHCl and 6 M-GuHCl plus 10 mM-DTT (Table 1). The only way in which the apparent M_r can exceed the true value is if the (second) virial coefficient in the presence of GuHCl is lower than in phosphate buffer alone. As this implies that aqueous GuHCl is a poorer solvent than phosphate buffer, i.e. that molecular interactions are enhanced in GuHCl, this is not a physically viable explanation.

Self-association or not?

Lipases from several sources have been investigated (Brockerhoff & Jensen, 1974; Macrae, 1983). Many are acidic glycoproteins of M_r between approx. 20000 and 60000 (Macrae &

Hammond, 1985). From the nature of lipase interaction with hydrophobic surfaces, it seemed plausible that self-association might play a part in lipase solution behaviour (Roberts & Tombs, 1987). However, from the recent high-resolution X-ray crystal structures of *M. meihei* lipase (Brady *et al.*, 1990) and human pancreatic lipase (Winkler *et al.*, 1990) and the M_r values in this paper, this seems unlikely both for the crystal and in solution.

A *C. viscosum* lipase was assigned an M_r of 27000 (Isobe & Sugiura, 1977) and *Ps. fluorescens* lipase 33000 (Sugiura & Oikawa, 1977), both obtained by using SDS/PAGE. Our values are similar, obtained by both calibrated SDS/PAGE and the absolute method of sedimentation equilibrium.

We conclude that the lipases of *C. viscosum* and *Pseudomonas* sp. do not self-associate in solution, and probably do not have substantial hydrophobic patches. Whether one is exposed when they enter the interface remains a matter for conjecture.

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