Biochimica et Biophysica Acta 902 (1987) 327–334
Elsevier

BBA 73662

Preparation of fluorescent derivatives of lipases and their use in fluorescence energy transfer studies in hydrocarbon / water interfaces

G.A. Roberts and M.P. Tombs

Unilever Research Laboratory, Colworth House, Sharnbrook, Bedfordshire (U K)

(Received 30 March 1987)

Key words Fluorescence energy transfer, Lipase, Hydrocarbon/water interface

Fluorescein isothiocyanate reacted with a chromobacter and pseudomonad lipase to yield mono-substituted, fully active, enzymes. With the carbocyanine dye 1,1′-dioctadecyl-3,3,3′,3′-tetramethylindocarbocyanine perchlorate (DiI) in the non-aqueous phase, fluorescence energy transfer was used to follow the lipase and similarly labelled model proteins in and out of the interface in heptane, and heptane / di-*O*-palmitoyl-*rac*-glycerol (a substrate analogue), emulsions. Competitive binding, and displacement by other proteins could also be followed.

Introduction

In previous work [1] we used the formation of complexes with concanavalin A as a probe of the ability of fungal lipases (EC 3 1 1 3) to enter and leave the water/triacylglycerol interface This is necessary information because the mechanism of lipases is dominated by their interfacial location It has always been difficult, to know how much of the enzyme present is actually in the interface, and therefore potentially active (since it must also be in the correct configuration) and how much remains in the bulk phase

Lipases are used in food processing, for example interesterification to make cocoa butter [2] In these systems the lipase is only one of the proteins present in the crude preparations used, and must compete for the interface with many other components This can seriously affect the efficiency of reactors [3], and we have no idea how much of the lipase present is actually effective

We had previously [1] concluded that lipases possessed no special properties which enabled them to compete particularly well for their place in the interface, but no direct measurements were available Indirect measurements of activity [4] confirmed that lipase inhibition was indeed due to competition for the interface, and not some other interaction

There was a need to follow lipases in and out of the interface in the presence of other proteins It seemed to us possible that a fluorescence method might be suitable Radiationless energy transfer takes place between a fluorescent donor and an energy acceptor at distances up to, but not beyond, about 6 nm [5] so that if we could attach one fluorescent label to the lipase, and position a second interacting one in the interface or in the non-aqueous phase, we should see the effect when the lipase is in the interface, but not otherwise Thus we could distinguish molecules in or near the interface from those in the main aqueous phase Lipases are about 3–4 nm in diameter [1] so that a

Abbreviations DiI, 1,1′-dioctadecyl-3,3,3′,3′-tetramethylindocarbocyanine perchlorate, FITC, fluorescein isothiocyanate

Correspondence M P Tombs, Unilever Research Laboratory, Colworth House, Sharnbrook, Bedfordshire MK 44 ILQ, U K

surface layer and possibly part of the next one should be detected

In 1977 Vaz et al [6] described a method where the approach of proteins to phospholipid membranes could be determined by using the intrinsic fluorescence of the protein to excite a fluorescent-labelled phospholipid. We have not exploited intrinsic fluorescence, since we wished to study lipases in the presence of other proteins However, the energy transfer method is clearly applicable to the problem

This paper describes results which suggest that a technique based on this approach can be used to determine surface occupancy by lipases in the presence of other proteins While the work was in progress Verger and co-workers [7] tackled somewhat similar problems, but attempted to solve the technical difficulty by ^{125}I radiolabelling of the lipase

Methods and Materials

Fluorescein-labelled proteins, ovalbumin (4 mol/mol), bovine serum albumin, α-lactalbumin and concanavalin A (all at about 1 mol/mol), fluorescein isothiocyanate and DiI (1,1′-dioctadecyl-3,3,3′,3′-tetramethylindocarbocyanine perchlorate) were from Molecular Probes Ltd , Eugene, OR, U S A Pseudomonad and chromobacter lipase were from Genzyme Ltd Di-*O*-palmitoyl-*rac*-glycerol and fluorescein-labelled dog albumin were from Sigma

Labelling A typical labelling reaction was as follows 10 mg of lipase in 5 ml 50 mM sodium phosphate (pH 7) was adjusted to pH 9–9 5 and 2 mg of fluorescein isothiocyanate (FITC) added The pH was held above 9 for 60 min at room temperature A pH above 9 was essential for success Then the labelled protein was separated from excess reagent by passage through Sephadex G-25, with transfer into 50 mM sodium phosphate (pH 7) Fluorescein showed anomalous retardation The solution of FITC-lipase was stable for at least 3 months at −20 °C

Characterisation The average labelling was found from absorbance of the substituted protein at 494 and 280 nm A molar absorbance of 58 000 at 494 nm was used to estimate fluorescein and A_{280}, was used for protein with a molar absorbance of 36 000 for the lipase It was necessary to correct the 280 nm absorbance for the fluorescein contribution This was found to be 0 4 × absorbance at 494 nm, by a separate measurement on fluorescein isothiocyanate solution in water The fluorescein group absorbance will vary with the environment, but this is unlikely to introduce significant errors By electrophoresis in discontinuous Tris-glycine buffers (pH 8 8) in 10% polyacrylamide gels by standard methods [8], the lipases were homogeneous After labelling at least 95% of the protein, as revealed by staining with coomassie blue, followed by scanning, migrated as a single fluorescent zone in the relative position expected for a single substitution In SDS-containing buffers the relative mobility also correspond to a single substitution No significant fluorescence appeared in the buffer front

Chromobacter lipase had a mol wt. of 33 900 and pseudomonad lipase 32 700

After substitution the enzymes retained full activity in a standard acidometric assay [9] (2000 μmol fatty acid/min per mg lipase, released from an olive oil emulsion) The p*I* by isoelectric focussing for the chromobacter lipase was 7 00 before substitution and 5 8 after For pseudomonad lipase values were 4 2 and 3 2, respectively

Fluorescence measurements These were always in 1 cm cuvettes, in a Baird-Novo II spectrofluorimeter

All measurements were at 18 °C for most experiments excitation was at 492 nm

The emission was strongly pH dependent though the exact curve varied with the protein pH 7 showed least pH dependence for the lipase, but albumin was quite different (Fig 1)

Preparation of heptane emulsions A typical emulsion contained 1 6 ml of 50 mM sodium phosphate (pH 7), 0 3 ml of 2% (w/v) gum arabic (Merck) and 0 1 ml of heptane (100–120 °C, petroleum ether) dispersed with an M S E sonicator, fitted with microtip, for 20 seconds In use this dispersion was diluted to 1 50 with appropriate buffer containing 0 3% (w/v) gum arabic Attempts to diminish gum arabic led to unstable emulsions

Where DiI was present it was dissolved (0 4 mg/ml) in the heptane before dispersal At this concentration molecules should be within 5 nm of

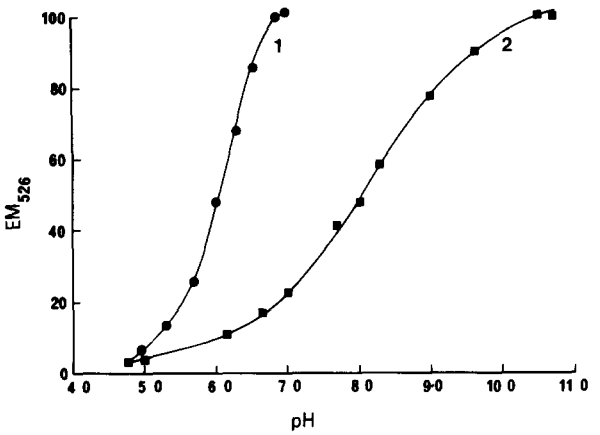

Fig 1 pH dependence of fluorescence of (1) FITC chromobacter lipase and (2) FITC bovine serum albumin 10 μg of labelled lipase was added to 4 ml of phosphate buffer between pH 5 and 7 Excitation was at 492 nm and emission at 526 nm

any point in the interface, even if it is not selectively concentrated into it

Surface area measurement Available surface area in the emulsion was measured by using a Quantimet image analyser, in conjunction with a microscope [10] This instrument processes a television camera image of the emulsion droplets and prints a number-size distribution plot, and on the reasonable assumption that the emulsion droplets were spherical, calculated the surface area of a known volume of heptane droplets

Because Brownian motion in the magnified image caused difficulties in defining the droplet diameters, we also used a particle counter (Coulter counter PA II) This instrument prints the same data, but is based on electrical conductivity measurements The two methods gave closely similar results

Energy transfer We use the relationship, derived by Forster [5]

$$R_0 = (JK^2Qn^{-4})^{1/6} 9\ 7\ 10^2 \text{ nm}$$

to estimate R_0 the distance at which energy transfer has a 50% probability where K is an orientation factor, taken to be 0 66, n is the refractive index, Q the quantum yield and J the overlap integral (cm^2/mol) The latter was estimated from fluorescein spectra in water and dil in heptane The dil spectrum varied considerably between heptane, dimethyl formamide and tributyrin but the heptane spectrum was used since most of our experiments were in heptane or heptane-triacetylglycerol mixtures Trial evaluations based on values for n of 1 33 or 1 5 and quantum yields from 0 1–0 7 gave a result for R_0 varying between 4 5 and 6 7 nm Using the refractive index of water and a quantum efficiency of 0 5 yielded 6 nm

Results

Model proteins

Fig 2 illustrates a typical titration in which aliquots of labelled protein were added to emulsions made with and without dil As expected, in the absence of dil the emission increased in a linear fashion, while in its presence fluorescein emission was quenched up to an apparent end point, after which it again increased in a linear fashion

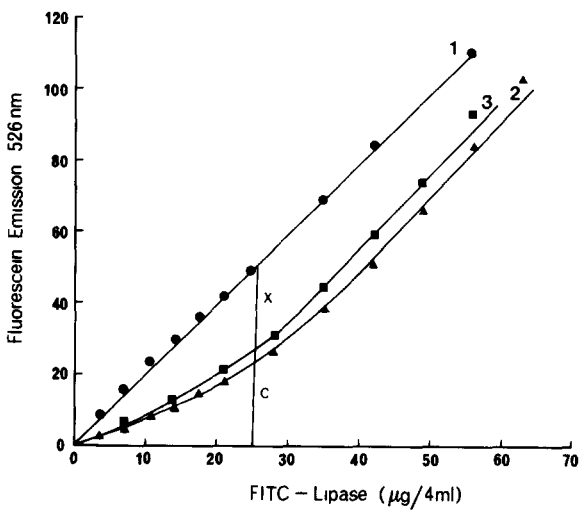

Fig 2 Titration of fluorescein-labelled chromobacter lipase into 4 ml of a heptane emulsion containing 1% (w/v) 1,2-di-*O*-palmitoylglycerol and dil (line 3) or heptane emulsion containing only dil (line 2) In contrast with line 1, where the heptane emulsion contained no dil, initially there was quenching of fluorescein fluorescence corresponding to the protein entering the interface and energy transfer occurring When the interface was full no further quenching was observed The difference between line 1, with no dil, and the other lines was used to estimate the ratio of free to bound protein In the example shown, the ratio of bound to free protein was X/C at a total added protein of 25 μg The end point of the titration, corresponding to saturation of the interface was taken as the point where X became constant

Because an emulsion was used there was a major scattering peak in the emission spectrum, which was minimised by using filters

Unlike previous experimental situations, where a receptor could be precisely located in a surface membrane [6] our receptor, dil, dissolved in the heptane droplets Thus only a small proportion of the dil molecules could show an enhanced emission by transfer from fluorescein in the interface

We did observe enhancement of the dil emission when fluorescein was present in the interface, but it was never more than 5% of the total emission There was also a small overlap with the fluorescein emission Measurement of the quenching of fluorescein associated with energy transfer (no quenching was observed in the absence of dil) was a much more sensitive way of following the surface interactions

Fluorescein labelled bovine serum albumin, dog albumin, α-lactalbumin and concanavalin A, all gave similar titration curves

Surface area estimation

Distributions of droplet size were skew unimodal, with mean diameters of the order of 0 8 μm Summing the radii led to area estimates of 6–7 m^2 per g of dispersed hydrocarbon, which corresponds to about 40 cm^2/ml emulsion Emulsions were stable for at least 2 h, and measurement before and after titration showed no significant difference

Lipases

At the levels of lipase we used the substrate was completely hydrolysed in seconds It was impossible to make measurements in a triacylglycerol emulsion, but data could be obtained for a solution of triacylglycerol in heptane In practice, before the first data point all the triacylglycerol would have been hydrolysed to yield fatty acid and possibly some monoacylglycerol

We tried to avoid this by inhibiting the enzyme Both diisopropyl fluorophosphate and, better, diethylnitrophenyl phosphate inhibited the enzyme but although the latter gave 99% inhibition, the residual activity was still too great

An alternative is illustrated in Fig 2, Ether-linked analogues of triacylglycerol are available, and are not attacked by the enzyme As Fig 2 and results in Table I indicate, there was little difference in the shape of the adsorption curve

Competition studies

Fig 3 shows that, even if the interface was initially filled with protein, (in the example shown, unlabelled ovalbumin), part of the interface, but not all, was accessible to labelled ovalbumin added subsequently About two thirds was inaccessible even at a high label to unlabelled ratio

A similar result was obtained when the surface was pre-loaded with serum albumin, and FITC-ovalbumin added

Preloading the interface with labelled protein allowed us to observe displacement by unlabelled molecules (Fig 4A) Sequential additions show displacement of label with a new equilibrium reached in about two minutes The presence of 1,2-di-*O*-palmitoylglycerol had little effect (Fig 4B)

Discussion

Labelling reaction

We were fortunate in labelling the lipases used, since we obtained an assembly of molecules each bearing one substituent We have no evidence that this single substitution was always in the same position, which would require extensive sequence

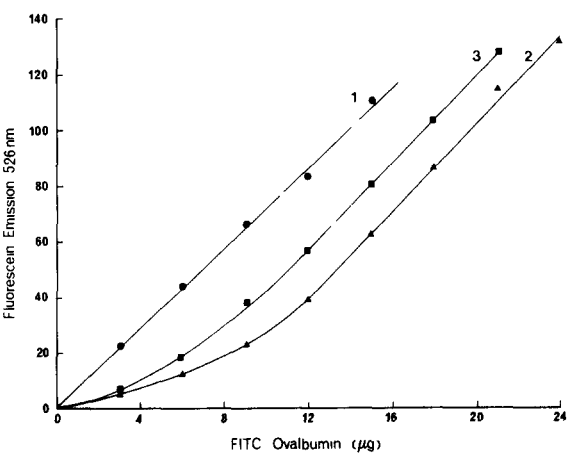

Fig 3 Titration of FITC ovalbumin into a heptane interface in the absence (1) and presence (2) of dil Curve (3) shows the effect of preincubating the emulsion (+ dil) with a quantity (12 μg) of unlabelled ovalbumin, sufficient to fill the interface

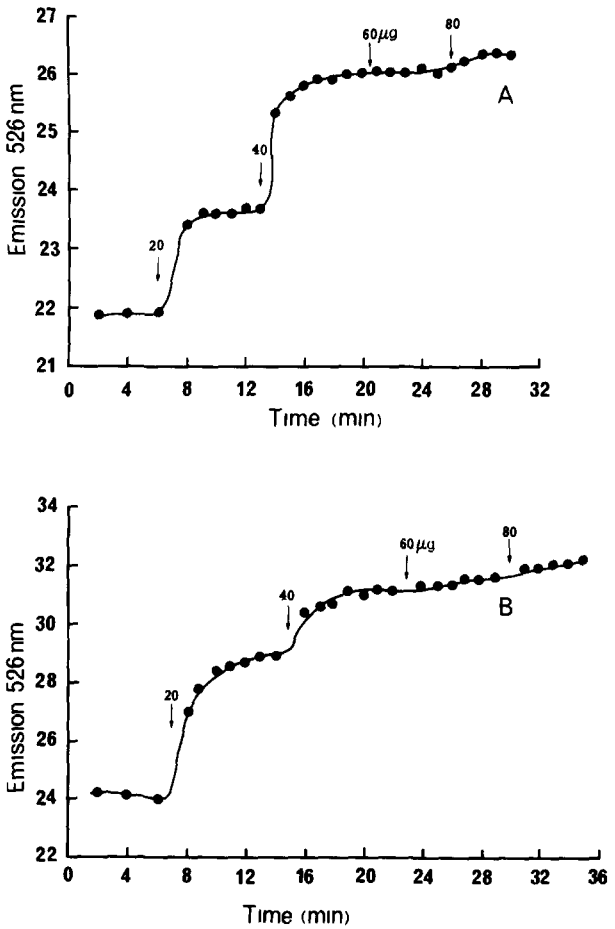

Fig 4 (A) Displacement of FITC chromobacter lipase by unlabelled lipase 4 ml of an emulsion of heptane containing dil quencher was preincubated for 20 min with sufficient FITC chromobacter lipase to fill the interface (7 μg ml^{-1}) Small aliquots of 10 μl of unlabelled lipase (2 μg ml^{-1}) were then sequentially added and the change in fluorescein emission (526 nm) followed with time (3) (B) The experiment was repeated using an emulsion of heptane containing 1% (w/v) 1,2-di-*O*-palmitoylglycerol all other conditions were identical

data No activity was lost as a result of the substitution Fungal and bacterial lipases are often isolated in nicked form, and since α-amino groups have a lower pK than lysine ϵ-amino groups, and the isothiocyanate reacts preferentially with the uncharged amino, the nicked ends and the N terminus label first With an *Aspergillus* and *Mucor miehei* lipase preparations this led to disintegration of the molecule into fragments which could not then be used An electrophoretic analysis after labelling would appear desirable, as a check on the integrity of the labelled enzyme Since the labelling reaction produces two charge differences from the unlabelled enzyme for each substitution it can also easily discriminate potential mixtures, such as mono- and di-substituted molecules

In our example all the molecules had a single substitution Absorbance measurements, which can only give values averaged over all the molecules, also gave a result of one

Emission spectra

The results illustrated in Fig 3, show that there was a radiationless energy transfer effect between fluorescein and the dil when lipases enter the interface The calculations detailed above point to a critical distance of about 6 nm

While we did observe some enhancement of dil emission in circumstances of energy transfer from fluorescein we have based our conclusions on measurement of the fluorescein emission spectrum for technical reasons explained above The problem then was that the magnitude, though apparently not the wavelength of the emission was very sensitive to pH, and lipase activity produces a pH change Buffers and careful pH monitoring were necessary

Interference from enzyme activity

Lipases are so effective that when the interface in a typical tributyrin emulsion was filled the entire emulsion was digested in a matter of seconds In heptane emulsions with dissolved triacylglycerols the results were effectively about monoacylglycerol and fatty acids in heptane They probably formed a surface layer on the emulsion It was not possible to measure lipase interaction on triacylglycerols at anything approaching saturation of the interface For this reason we made some measurements on a substrate analogue where glycerol ester links were replaced by ether links

Another possible approach, by inhibiting the enzyme, failed, since we could not achieve total inhibition

Adsorption isotherm

The ratio of protein bound (X) at a free con-

centration (C) can be estimated from plots of the type illustrated in Fig 2 If A is the surface area, K and z are constants, then the Langmuir adsorption isotherm can be written

$$\log X - \log A = \frac{1}{z} \log C + \log K$$

so that a value for $1/z$ can be obtained from a plot of log X against log C Fig 5 shows that such a plot is, as required, linear up to about 70% saturation of the interface This form of the isotherm does not hold at high surface occupancy The constant z can be used to measure the tendency to be in the interface since a low value corresponds to a higher proportion of protein in the interface By this criterion the lipases and bovine albumin enter the interface more than α-lactalbumin, concanavalin A or ovalbumin (Table I) at the same total protein concentration

Surface area occupancy

From plots of the type illustrated in Fig 2 it was possible to estimate the maximum entry of protein to within 6 nm of the interface, making the assumption that all fluorescein emission within this region is supressed

Transfer efficiencies ranging from 20% to 100% have been cited [5] We observed at least 90% suppression of fluorescein emission on adding dil,

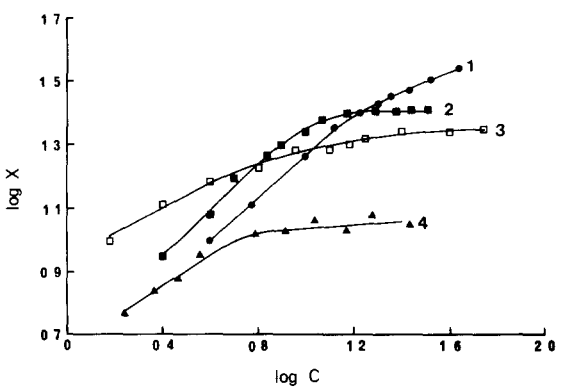

Fig 5 Langmuir adsorption isotherms for FITC derivatives of (1) chromobacter lipase, (2) bovine serum albumin, (3) ovalbumin, (4) canine albumin X and C are the amount of protein bound and free, respectively Values for the initial slope were used to obtain z (see Table 1) All determinations were on heptane emulsions

under conditions where nearly all the fluorescein would have been expected to be in the interface

Area estimation showed a range from 39–45 cm^2/ml of emulsion and these were used to give upper and lower estimates for the surface area available per molecule shown in Table I

Completely close packed monolayer arrays would require areas ranging from about 10 nm^2 for α-lactalbumin to 25 nm^2 for concanavalin A, if the molecules retained their globular configurations with dimensions similar to those in solution The areas found are considerably larger, but it should be noted that even so, the average distance between molecules was still only between one and two molecular diameters Previous values, from surface pressure measurements on air/water interfaces have given values far smaller than these [11] and cannot represent a monolayer condition

The ideas of multiple layer binding and partial unfolding were clearly set out by James and Augenstein in 1966 [12] while in 1979 Graham and Phillips [13] produced a substantial study of adsorption at a water/hydrocarbon interface, using radiolabelling and ellipsometry to find surface concentrations They found a primary layer of about 5 nm depth, and with lysozyme an area of 6 2 nm^2, for β-casein 19 2 nm^2 and for albumin 35 3 nm^2 can be calculated from their data This primary layer was irreversibly adsorbed but they

TABLE I

SURFACE INTERACTION CONSTANTS

z is derived from the absorption isotherm (see text) The area per molecule is estimated from the apparent maximal binding (see for example Fig 2) and surface areas in the range 39 3 cm^2/ml–45 cm^2/ml

Protein	Emulsion	z	Area per molecule (nm^2)
Lipase (Chromobacter)	heptane	1 6	46– 53
Lipase (Chromobacter)	heptane-di-*O*-palmitoylglycerol	1 5	60– 69
Lipase (*Pseudomonas*)	heptane	1 4	19– 21
Ovalbumin	heptane	2 7	116–133
Bovine albumin	heptane	1 5	143–164
Canine albumin	heptane	2 0	172–197
α-Lactalbumin	heptane	2 7	30– 34
Concanavalin A	heptane	2 5	237–272

also found that further, reversible, adsorption took place to give layers up to three molecules deep There was some evidence both in this study and others [11] to suggest that the primary layer undergoes partial unfolding, though the extent depended on the individual protein

Partially unfolded molecules would occupy a larger surface area than compact spheres, which may be the reason for the relatively large areas found in this work

As shown in Fig 7 there is a relationship between the molecular radius and the area occupied and, there must be some size-dependent interaction between the molecules in the interface to produce this result

Reversibility of absorption

After the interface had been filled with labelled lipase, some of it could be displaced by adding unlabelled lipase A simple statistical replacement should follow

$$C = \frac{K\ R}{R+1}$$

where C is the free concentration of labelled protein, and R is the mole ratio (unlabelled/labelled protein) for the total protein in the system Since the concentration was measured in arbitrary fluorescence units, K is an arbitrary constant

Fig 6 shows that the relevant plot for lipase was linear up to a ratio of 1 0 Deviation afterwards might indicate that a proportion of the initial layer was very difficult to displace

This was also the case when the emulsion was pre-loaded with unlabelled protein The take up of labelled protein added subsequently was reduced, but not completely prevented

The displaced protein must have been within 6 nm of the interface initially, and must therefore have been in either a primary or a secondary layer

At present we cannot distinguish between these two possibilities, though the model provided by Graham and Phillips [13] and James and Augenstein [12] is only compatible with displacement from a secondary layer

Our results fit this model for the adsorption process, but they have some implications for the activity of lipases It is difficult to see how a primary layer of lipase molecules could retain

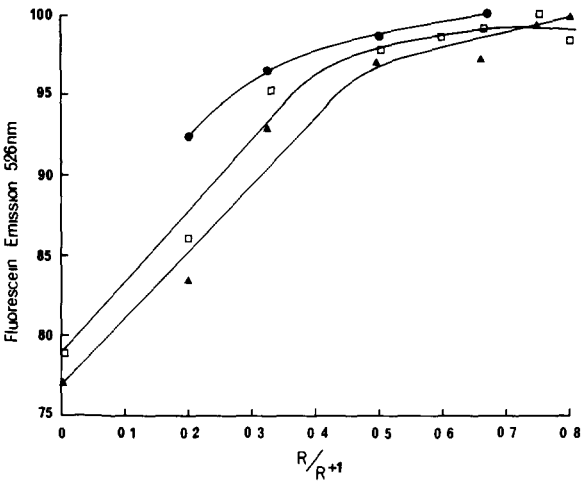

Fig 6 Plot of emission, which is a measure of free labelled protein, against $R/R+1$ where R is the ratio of unlabelled/labelled protein in a heptane emulsion to which varying mixtures of labelled and unlabelled protein have been added (see text) Three separate experiments are shown, all with ovalbumin

activity if they are partially unfolded Also, lipases have no special structural features such as intrachain disulphides, nor do they have unusual stability, which might help to resist surface forces [1]

Lipases must normally function in the presence of other proteins which can obstruct their access to the necessary position in the interface but it is

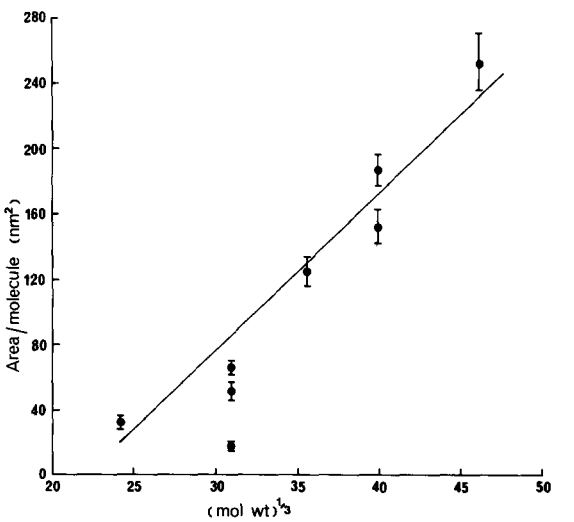

Fig 7 Relationship between area/molecule and molecular weight for proteins listed in Table I A linear relationship would be expected for spherical molecules, where the molecular size has a direct bearing on the area occupied

possible, that some of these might also protect against surface unfolding (In the emulsion used, gum arabic might also modify the hydrocarbon/water surface) Even if lipase alone is present, an extended flattened chain is a relatively open structure and occupies so much interface that only a proportion of added protein would needed to fill it, so that the remainder could form a second layer, retaining its structural integrity, but still able to make contact with the substrate Thus lipases might preferentially bind to partially unfolded protein already in the interface, rather than the free surface

Lipases in solution undergo self-association [1] as would be expected of proteins with hydrophobic patches If we imagine lipase dimers striking the interface then this becomes a plausible model Some such mechanism is needed to explain how lipases avoid what seems to be an otherwise inevitable unfolding process

The fluorescence based technique described here offers a novel and independent method for investigating enzymes in oil-water interfaces

Acknowledgements

We are grateful to Dr P Garland for his advice and encouragement during this work We also thank Dr R A Badley for help with critical distance calculations

References

1 Tombs, M P and Blake, G G (1982) Biochim Biophys Acta 700, 81–89
2 Macrae, A R and Hammond, R C (1985) Biotechnol Genet Eng Rev 3, 193–217
3 Wisdom, R A , Dunhill, P and Lilly, M D (1985) Enz Microb Technol 7, 567–572
4 Gargouri, Y , Pieroni, G , Riviere, C , Sugihara, A , Sarda, L and Verger, R (1985) J Biol Chem 260, 2268–2273
5 Stryer, L (1978) Annu Rev Biochem 47, 819–46
6 Vaz, W L C , Kaufmann, K and Nicksch, A (1977) Anal Biochem 83, 385–393
7 Gargouri, Y , Pieroni, G , Riviere, C , Sarda, L and Verger, R (1986) Biochemistry 25, 1733–38
8 Hames, B D (1981) in Gel Electrophoresis of Proteins, (Hames, B and Rickwood, D , eds), IRL Press, Oxford
9 Desnuelle, P (1972) in The Enzymes, Vol 7, 3rd Edn Academic Press, New York
10 Bradbury, S (1983) J Microsc , 131, 203–210
11 MacRitchie, F (1978) Adv Protein Chem 32, 283–326
12 James, L K and Augenstein, L G (1966) Adv Enzymol 28, 1–36
13 Graham, D E and Phillips, M C (1979) J Colloid Interface Sci 70, 430–414, 415–426, 427–439