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A Continuous Assay for Lipases in Reverse Micelles Based on Fourier Transform Infrared Spectroscopy

Peter Walde and Pier Luigi Luisi*
Institut für Polymere, Eidgenössische Technische Hochschule, CH-8092 Zürich, Switzerland
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ABSTRACT: A new, simple, and fast method is described for measuring the lipase-catalyzed hydrolysis of triglycerides in reverse micelles. The course of hydrolysis can be followed by recording the Fourier transform infrared spectrum of the entire reaction mixture as a function of incubation time. Due to the presence of isosbestic points, the kinetics of the reaction can be followed at different regions of the spectrum, for example, in the stretching band region of carbon–oxygen double or single bonds (between 1750 and 1710 cm^{-1} or around 1160 cm^{-1}, respectively). It is shown that the method can be applied for determining the extent of hydrolysis and for the determination of the lipolytic enzyme's fatty acid specificity within the class of triglycerides.

There is a growing interest in lipases not only for their applications in fat hydrolysis and synthesis under mild conditions (Linfield et al., 1984) but also for their use as catalyst for a variety of reactions, e.g., aminolysis, thiotransesterifications, and oximolysis. One of the problems in the enzymology of lipases is the lack of a simple spectrophotometric assay with triglyceride substrates. The reason lies in the poor water solubility of both synthetic and natural substrates.

The use of reverse micelles can in principle overcome these difficulties. In fact, one of the peculiarities of enzymes in reverse micelles (or "water-in-oil microemulsions" at higher water content) is their capability of being active with substrates that are present in the oil (organic solvent) phase [for reviews see Luisi (1985), Martinek et al. (1986), Waks (1986), and Luisi and Steinmann-Hofmann (1987)]. Generally, in a reverse micelle solution, the enzyme is solubilized within the aqueous core of the micelle and protected against unfavorable contacts with the organic solvent by a layer of surfactant molecules (Martinek et al., 1986; Luisi et al., 1988). In clear contrast to those cases where enzyme powders are directly dispersed in organic solvents without surfactants (Zaks & Klibanov, 1985), reverse micellar solutions are stable and optically transparent systems, allowing one to carry out spectroscopic studies. Several previous papers have considered phospholipases/lipases in reverse micelles (Misiorowski & Wells, 1974; Malakhova et al., 1983; Fletcher et al., 1985,

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1987; O'Connor et al., 1986; Han & Rhee, 1985, 1986; Han et al., 1987a,b; Hochkoeppler and Palmieri, unpublished results), the last two of these being mainly concerned with the problem of the assay.

In the present paper, we describe a new, simple, and fast method for measuring the lipase-catalyzed hydrolysis of triglycerides in reverse micelles. The course of hydrolysis has been followed by recording the Fourier transform infrared spectrum of the entire reaction mixture as a function of time. The kinetics of the reaction can be followed at different regions of the spectrum: either in the stretching band region of the carbonyl group (1710–1750 cm^{-1}) or the carbon–oxygen single bond region (around 1160 cm^{-1}) or the O–H stretching band section (around 3500 cm^{-1}). It will be shown that the method can be applied for determining the extent of hydrolysis and substrate specificity. In the present paper the investigation of specificity is restricted to the class of triglycerides in which the three fatty acid groups are equal.

Our approach offers an analytical way to study the behavior of water during an enzymatic reaction. This is particularly interesting for hydrolases, where water is one of the substrates, and may offer a general method to investigate the behavior of enzymes during consumption of water.

Materials and Methods

Reagents. AOT was a product from Sigma and was used as obtained. Newer batches of Sigma AOT[1] are of satisfactory purity (Aveyard et al., 1986; Fletcher et al., 1987). The determined dimensions of the AOT micelles that we prepared in the present study were in good agreement with published data (see below). Isooctane (for UV spectroscopy) was from Fluka. Trihexanoylgycerol (Tricaproin), trioctanoylglycerol (tricaprylin), tridecanoylglycerol (tricaprin), tritetradecanoylgycerol (trimyristin), trihexadecanoylglycerol (tripalmitin), hexanoic acid (caproic acid), octanoic acid (caprylic acid), sodium octanoate (sodium caprylate), decanoic acid (capric acid), tetradecanoic acid (myristic acid), hexanoic acid ethyl ester, decanoic acid ethyl ester, and tetradecanoic acid ethyl ester were of the highest purity available from Fluka (≥97%). The vegetable oils from sunflower, olive, linseed, and jojoba were from the local market. Porcine pancreatic lipase (type VI-S) was from Sigma and the fungal *Rhizopus delemar* lipase from Fluka. All other lipases were a gift from Amano Pharmaceutical Co. Ltd. (Germany): lipase AP6 (from the fungus *Aspergillus niger*), bacterial lipase P (from the bacteria *Pseudomonas fluorescens*), and lipase AY (from the yeast *Candida cylindracea*, recently renamed *Candida rugosa*). Analytical SDS–polyacrylamide ge electrophoresis showed that all lipase samples were rather heterogeneous. They consist of different proteins.

Quasi-Elastic Light Scattering. Quasi-elastic light scattering measurements were carried out at 25 °C with an apparatus described elsewhere (Vancso et al., 1988). Hydrodynamic radii of AOT reverse micelles can be determined from a cumulant analysis of the intensity autocorrelation function (Koppel, 1972) with a reproducibility of ±5%.

Time-Resolved Fluorescence Quenching. The fluorescence probing measurements were carried out at 25 °C by Dr. Jaques Lang at the Institut Charles Sadron in Strasbourg, France. The fluorescence probe used was ruthenium tris(bipyridyl) chloride and potassium ferricyanide as quencher. It has been shown that the magnitude of the intramicellar quenching of the probe is proportional to the quencher concentration and inversely proportional to the micelle concentration (Lang et al., 1988). The method allows the determination of the concentration of the reverse micelles. On the basis of this value, the number of surfactant molecules per micelle can be calculated on knowing the concentration of free, nonassociated surfactant molecules (which is approximately the critical micelle concentration). The radius of the water pool can then easily be calculated on assuming monodispersity of the spherical micelles and on knowing the water/hydrocarbon interface area covered by one surfactant molecule (Lang et al., 1988). The experimental accuracy for the determination of the surfactant aggregation number is ±15%.

For comparison with light scattering data, the hydrodynamic radius can be estimated in a first approximation as the sum of the water pool radius and the length of the extended surfactant molecule.

FTIR Spectroscopy. All Fourier transform infrared spectra were recorded on a Nicolet 5SXC FT-IR spectrometer, using a CaF_2 cell from Wilmad with a fixed pathlength of 0.01 cm. Thirty-six scans were taken of each sample at a resolution of 4 cm^{-1}. The time required for recording one scan was approximately 1 s. The determined molar extinction coefficients are given with their standard deviations.

Preparation of the Reaction Mixtures and Standard Solutions. First of all, the lipase was dissolved in an aqueous buffer solution. A 0.1 M sodium acetate, pH 5.6, buffer was used in all cases, except with porcine pancreatic lipase where 0.1 M Tris-HCl was applied with pH 7.5. Enzyme stock solutions were prepared daily and stored before use at 4 °C in the refrigerator. In the case of *R. delemar* lipase the stock solution was generally 10 mg/mL; in the other cases it was 1 mg/mL.

An appropriate amount of buffer and/or lipase stock solution was added to 1 mL of 100 mM AOT in isooctane by a microsyringe to give the desired w_0 value and enzyme concentration. w_0 stands for the ratio of molar concentration of water to the molarity of surfactant ($w_0 = [H_2O]/[AOT]$). After solubilization of the buffer ions and the enzyme by shaking and vortexing at room temperature for about 2 min (until a clear solution was obtained), the reaction was started by adding 1 mL of a substrate solution in isooctane (typically 100 mM triglyceride). The reaction mixture was kept in a water bath at 25 °C without stirring, and samples of approximately 200 μL were withdrawn at a desired time and analyzed by FTIR spectroscopy. The reaction mixtures could also be analyzed continuously by using a thermostated cell. Typical experimental conditions for the reactions were as follows: 50 mM AOT in isooctane, $w_{0,st} = 11.1$, added buffer = 0.1 M sodium acetate (or Tris), pH 5.6 (or 7.5), with an overall lipase concentration of 50 μg/mL. Control experiments showed that only negligible amounts of fatty acids are released in the absence of lipase within the time scale used in our study. All measurements for calibration purposes were carried out in a similar way with the exception that no enzyme was added. In all the cases where spectra have been recorded in the absence of AOT, pure isooctane instead of a 100 mM AOT/isooctane was used.

The reproducibility of preparing the solutions was checked by using the same 100 mM AOT stock solution and preparing nine AOT solutions, each containing 50 mM surfactant with $w_0 = 11.1$. The standard deviation of the measured infrared absorption value at 3457 cm^{-1} was ±2% and at 1735 cm^{-1} ±1%. Note that the experimental errors made with the mi-

[1] Abbreviations: AOT, sodium bis(2-ethylhexyl) sulfosuccinate; FTIR, Fourier transform infrared; FA, fatty acid; FAE, fatty acid ester; TG, triglyceride; 18:3, numerical symbol form for fatty acids (18 carbon atoms with 3 double bonds); $w_0 = [H_2O]/[AOT]$.

Table I: Size of AOT Reverse Micelles in Isooctane at 25 °C As Determined by Light Scattering and Fluorescence Probing[a]

system	light scattering R_H (Å)	fluorescence probing [M] (mM)	fluorescence probing R_{wp} (Å)	literature data R_H (Å)	literature data R_{wp} (Å)
50 mM AOT, w_0 = 11.1	33	0.41	21	35[b]	20–22[c]
50 mM AOT, 50 mM trioctanoylglycerol, w_0 = 11.1	32	0.38			
50 mM AOT, 100 mM octanoic acid, w_0 = 11.1	31	0.60			

[a] R_H and R_{wp}: hydrodynamic and water pool radii, respectively. [M]: concentration of the reverse micelles. [b] From Eicke (1982), light scattering. [c] From Maitra (1984), nuclear magnetic resonance; and Pileni et al. (1985), small-angle X-ray scattering.

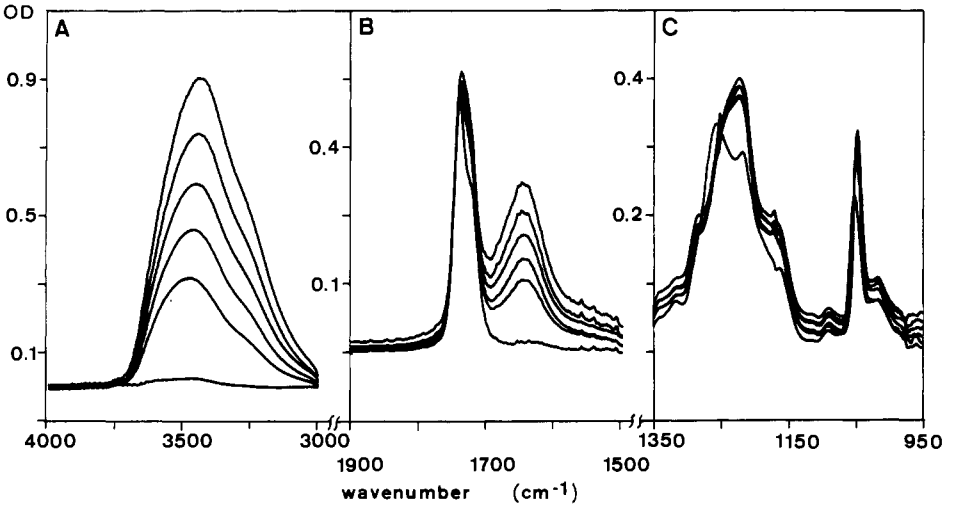

FIGURE 1: FTIR spectrum of 50 mM AOT in isooctane between 4000 and 3000 cm^{-1} (A); 1900 and 1500 cm^{-1} (B); and 1350 and 950 cm^{-1} (C). Path length: 0.01 cm. The increases in optical density correlate with the amount of added water: w_0 = 0, 5.6, 8.3, 11.1, 13.9, and 16.7.

crosyringe are reflected mainly at 3457 cm^{-1}, in the O–H (st) region of water; see below.

Thin-Layer Chromatography. Thin-layer chromatography plastic sheets covered with a 0.2-mm layer of silica gel (60 F_{254}) were obtained from Merck. The solvent mixture used was hexane/diethyl ether/acetic acid (50:50:1 v/v/v), and the spots were stained with a 0.1% solution of 2′,7′-dichlorofluorescein in methanol, viewed under ultraviolet light at 366 nm.

Most of the reactions carried out were analyzed qualitatively by thin-layer chromatography. In all the cases, apart from reaction products (mono- and diglycerides and fatty acids), nonhydrolyzed triglycerides could be detected. This result is in agreement with the observation made that the reaction yields never reached 100% under the conditions used; see below. In the case of the trioctanoylglycerol hydrolysis, the following R_f values for the different compounds in the reaction mixture were obtained: 0.0 (AOT), 0.04 (monooctanoylglycerol), 0.16 (dioctanoylglycerol), 0.36 (octanoic acid), and 0.54 (trioctanoylglycerol).

Results

Physical Characterization of the Reverse Micellar System. Unless otherwise stated, all activity measurements described below were carried out in isooctane in the presence of 50 mM AOT with an initial w_0 value of 11.1 ($w_{0,st}$). Let us therefore first briefly describe the physical properties of this reverse micellar system. Since we will be working with micelles containing a significant amount of substrate, it is possible that the physical properties of the micelles (e.g., dimensions, aggregation numer, thermodynamic stability) are influenced by the presence of substrate molecules. There are in principle three different possible sites of localization of these compounds, namely, in the bulk organic phase, at the micelar interface, or in the water pool of the micelles.

To characterize the micelles with and without triglycerides, the methods applied were quasi-elastic light scattering, Fourier transform infrared spectroscopy (FTIR), and fluorescence probing (see Materials and Methods). Although this was already known from literature, we first determined as a control the mean hydrodynamic and water pool radius of the AOT reverse micelles used in this study. With the light scattering method, we obtained a mean hydrodynamic radius of 33 Å for 50 mM AOT and w_0 = 11.1; see Table I. The concentration of micelles in this solution was determined by time-resolved fluorescence probing to be 0.41 mM. Each micelle is therefore built up on average by 120 AOT molecules [assuming a critical micelle concentration of 0.6–0.9 mM (Jean & Ache, 1978)]. On the basis of the determined aggregation number, the water pool radius of the reverse micellar spheres has been calculated to be 21 Å [water/hydrocarbon interface covered by one AOT molecule at w_0 = 11.1 assumed to be 47 $Å^2$ (Eicke & Kvita, 1984)]; see Table I. If we add to the estimated water pool radius of 21 Å the length of an extended AOT molecule of 12 Å (Zulauf & Eicke, 1979), we obtain the same value as obtained for the hydrodynamic radius from the light scattering measurements, 33 Å. The results of the two independent measurements are therefore consistent and also are in good agreement with literature data (Eicke, 1982; Maitra, 1984; Pileni et al., 1985), Table I.

In addition to the two methods described above with which we obtained information on the number of micelles and their size, we measured the FTIR spectrum of 50 mM AOT in isooctane as a function of added water (w_0 = 5.6–16.7); see Figure 1. Infrared spectroscopic measurements allow us, for example, to study the properties and the behavior of water molecules inside the water pool of reverse micelles. Recently, such a study has been carried out for water/AOT in heptane, carbon tetrachloride, or toluene (MacDonald et al., 1986). Generally, the published spectra of AOT in heptane are comparable with our measurements of AOT in isooctane. For the present work we primarily wanted to know whether and to what extent the entire infrared spectrum of AOT is dependent on the amount of water present, because the water

concentration changes during the lipase-catalyzed reaction (see below).

For the sake of clarity, one can divide the spectrum into three regions. In Figure 1A, the range between 4000 and 3000 cm^{-1} is shown, where the spectrum is dominated by the O–H stretching bands of water (peak maximum at 3457 cm^{-1}). On plotting the optical density at 3457 cm^{-1} as a function of w_0, a straight line is obtained (data not shown). From the intercept on the ordinate the presence of small amounts of water in the AOT sample used could be detected (0.7 water molecule per AOT). A straight line is also obtained when the integral between 4000 and 3000 cm^{-1} is plotted against w_0. The molar extinction coefficient (ϵ) of micellar water at 3457 cm^{-1} has been determined to be 104 ± 6 M^{-1} cm^{-1} (w_0 = 5.6–16.7).

In Figure 1B, the carbonyl stretching bands, C=O (st), of the two carbonyl groups of the AOT molecule around 1735 cm^{-1} and the bending mode (ν_2) of the added water (around 1643 cm^{-1}) are shown. If we plot the integral between 1900 and 1500 cm^{-1} as a function of w_0, a straight line is obtained, indicating that the C=O (st) band intensity does not vary significantly with increasing water concentration, at least within the range studied ($5.6 \leq w_0 \leq 16.7$). This observation is important for the quantitative analysis of the triglyceride hydrolysis outlined below. Note that the effect of the added water on the C=O (st) band shape and intensity around 1735 cm^{-1} is mainly due to an overlapping of the band arising from the ν_2 mode of water, centered around 1643 cm^{-1}. Therefore, no significant hydration of the carbonyl groups seems to occur.

The range of the infrared spectrum of AOT reverse micelles between 1350 and 950 cm^{-1} is shown in Figure 1C. This region is rather complex because it is composed of different bands arising from various vibrational modes of the sulfonate head group and the carboxylic acid ester bonds of the surfactant. In particular, the sharp band around 1050 cm^{-1} arises from the symmetrical S=O stretching mode (MacDonald et al., 1986). The changes in this part of the spectrum were large on going from w_0 = 0 to 5.6, reflecting hydration of the polar head group of the surfactant molecules. Above w_0 = 5.6, the water effect is minor, indicating that head group hydration is completed.

Effect of Triglycerides on AOT Reverse Micelles. The presence of 50 mM trioctanoylglycerol did not change either the determined hydrodynamic radius or the micelle concentration significantly as indicated by light scattering and fluorescence probing measurements, Table I. Therefore, we conclude that most of the triglycerides are located in the bulk oil phase under the conditions used. This is supported by the observation that the entire infrared spectrum of trioctanoylglycerol as well as two other synthetic triglycerides tested (C_6 and C_{10}) and the vegetable triglyceride oils is not affected by the presence of AOT (data not shown). In all cases, the position of the C=O (st) peak maximum is at 1751 cm^{-1}. Molar extinction coefficients at this wavenumber have been determined for trihexanoylglycerol, trioctanoylglycerol, and tridecanoylglycerol to 1807 ± 36, 1678 ± 34, and 1722 ± 34 M^{-1} cm^{-1}, respectively. The corresponding values at 1715 cm^{-1} are 80 ± 2 M^{-1} cm^{-1} (for trihexanoylglycerol), 58 ± 2 M^{-1} cm^{-1} (for trioctanoylglycerol), and 68 ± 2 M^{-1} cm^{-1} (for tridecanoylglycerol).

The spectrum in the range between 1350 and 950 cm^{-1} of trioctanoylglycerol, for example, is characterized by three peaks, a broad one around 1230 cm^{-1}, a sharp at 1161 cm^{-1}, and one at 1100 cm^{-1} with a shoulder at 1111 cm^{-1}. These bands arise from asymmetric and symmetric C–O (st) vibrations of the triglyceride molecule. The molar extinction coefficients at 1161 cm^{-1} of three trglycerides have been determined: 905 ± 18, 772 ± 17, and 649 ± 17 M^{-1} cm^{-1} for trihexanoyl-, trioctanoyl-, and tridecanoylglycerol, respectively. In the case of tridecanoylglycerol, the peak maximum was found to be at 1154 cm^{-1} (ϵ = 719 ± 17 M^{-1} cm^{-1}), independent of the presence of AOT.

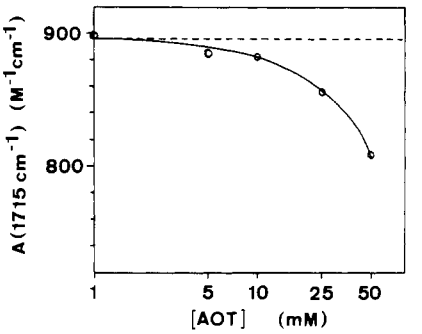

FIGURE 2: Effect of AOT concentration on the FTIR spectrum of decanoic acid at 1715 cm^{-1}. *A* stands for the molar absorbance [optical density × (path length)$^{-1}$ × (concentration)$^{-1}$] of the difference spectrum (decanoic acid in AOT/isooctane − AOT/isooctane). [Decanoic acid] = constant = 150 mM; w_0 = constant = 11.1; the dotted line shows the value obtained in isooctane alone (no AOT).

Effect of Fatty Acids on AOT Reverse Micelles. In contrast to the triglycerides, fatty acids clearly partition between the micellar interface and the bulk isooctane [where they exist probably in form of dimers (Ganguly & Mohan, 1988)]. This has been shown by measuring the micelle concentration by time-resolved fluorescence probing. The micelle concentration increases to 0.60 mM in the presence of 100 mM octanoic acid in 50 mM AOT/isooctane, w_0 = 11.1. The hydrodynamic radius is only slightly decreased to 31 Å, as determined by light scattering; see Table I.

A fatty acid partitioning is also indicated by the infrared spectrum. We recorded spectra of different fatty acids in isooctane alone or in the AOT reverse micellar system. Generally, if we compare the difference spectra—in which the AOT contribution has been subtracted from the measured spectrum—with the fatty acid spectrum in pure isooctane, clear differences are apparent at all fatty acid concentrations used (25–150 mM). These differences are most obvious in the C=O (st) peak area. While the peak position is independent of the presence of AOT at 1715 cm^{-1}, the intensity at this peak maximum is in the case of the difference spectrum significantly lower, and the peak is broadened to higher wavenumber. Both in pure isooctane solution and in micellar solution a linear relationship exists between the measured optical density and the fatty acid concentration. On the basis of such calibration curves for the difference spectra, we determined molar extinction coefficients for hexanoic acid, octanoic acid, decanoic acid, and tetradecanoic acid: 758 ± 32, 806 ± 26, 784 ± 36, and 781 ± 38 M^{-1} cm^{-1}, respectively.

As shown in Figure 2, the observed differences in intensity at 1715 cm^{-1} depend on the AOT concentration: the more AOT at constant w_0 (which means the more micelles), the greater the total amount of fatty acids at the interface and the larger the observed difference. If we look at the O–H (st) of the fatty acids above 3000 cm^{-1}, the effect of AOT is again apparent. First of all, the maximum of the fatty acid O–H (st) in pure isooctane lies around 3041 cm^{-1}, by using a fatty acid concentration between 25 and 150 mM. On recording the difference spectrum (fatty acid/AOT minus AOT), the intensity at 3041 cm^{-1} decreases while a new peak appears around 3500 cm^{-1}. The intensity of the new peak (at a constant fatty acid concentration) depends on the AOT concen-

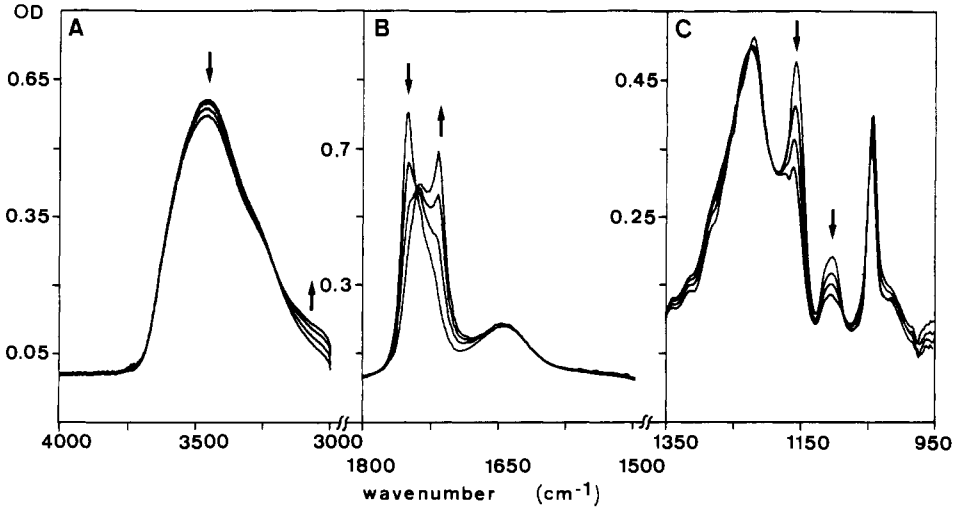

FIGURE 3: *R. delemar* lipase catalyzed hydrolysis of trioctanoylglycerol in AOT reverse micelles: time course of the FTIR spectrum between 4000 and 3000 cm^{-1} (A), 1800 and 1500 cm^{-1} (B), and 1350 and 950 cm^{-1} (C). AOT (50 mM) in isooctane, $w_{0,st}$ = 11.1, $[trioctanoylglycerol]_0$ = 38 mM, $[lipase]_{0v}$ = 0.05 mg/mL, T = 25 °C, path length = 0.01 cm. Incubation times: 0, 6, 20, and 90 min.

tration. The higher the AOT concentration, the more intense the new peak, and at the same time, the lower the intensity of the band at 3041 cm^{-1}. All this clearly indicates a fatty acid partitioning between the bulk isooctane and the reverse micellar aggregates.

Lipase-Catalyzed Hydrolysis of Triglyceride. As mentioned in the introduction, lipase assays are problematic because of the poor solubility in water of the substrates. In order to solubilize them in the solution that contains the enzyme, they are usually emulsified, and the resultant solution is then unsuitable for spectrophotometric analysis, so that discontinuous methods must be applied (Dennis, 1983; Brockman, 1984). The reproducibility of kinetic runs with emulsions is particularly difficult to attain, also because the stability of emulsions is low and depending upon the method of preparation.

We have mentioned that in order to tackle this problem, lipase assays in reverse micelles have been proposed. In particular, Rhee and co-workers presented an assay which is based on a colorimetric determination of the free fatty acids (Han et al., 1987a). More recently, Hochkoeppler and Palmieri introduced a continuous lipoxygenase-coupled spectrophotometric lipase assay which is however limited to triglyceride substrates containing linoleic acid (Hochkoeppler and Palmieri, unpublished data).

The use of FTIR permits a solution to the problem. The reaction under study will be the hydrolysis of different defined triglycerides and natural triglyceride mixtures (vegetable oils) catalyzed by lipases. The kinetics of a triglyceride hydrolysis in a AOT reverse micellar system can be followed by recording the entire FTIR spectrum of the reaction mixture as a function of time. A typical example is illustrated in Figure 3. During the course of the reaction, changes occur in five different regions of the spectrum: (1) a decrease in intensity around 3457 cm^{-1}, due to the decrease in the water concentration during the hydrolysis (Figure 3A); (2) an increase in intensity around 3020 cm^{-1}, mainly due to the production of free fatty acids (Figure 3A); (3) a decrease in intensity around 1751 cm^{-1} as a result of the decrease in fatty acid ester concentration (Figure 3B); (4) an increase in intensity around 1715 cm^{-1}, due to the formation of free fatty acids (Figure 3B); (5) a decrease in intensity around 1161 cm^{-1}, due to the decrease in fatty acid ester concentration (Figure 3C).

For a quantification of the reaction, we mainly looked at the spectral changes occurring between 1800 and 1500 cm^{-1}. Due to the presence of an isosbestic point at 1740 cm^{-1}, we can approximate the entire time course of the reaction with a simple conversion from compound A (with its peak maximum at 1751 cm^{-1}) to compound B (with its peak maximum at 1715 cm^{-1}). Compound A stands in our case for the fatty acid ester and compound B for the liberated fatty acid. On the basis of Beer's law and the molar extinction coefficients for A and B, it is possible to determine the concentrations of the two compounds at any time during the reaction. The molar extinction coefficient difference at 1715 cm^{-1} is therefore

$$\Delta\epsilon(1715\ cm^{-1}) = \epsilon_{FA}(1715\ cm^{-1}) - \epsilon_{FAE}(1715\ cm^{-1}) \approx \epsilon_{FA}(1715\ cm^{-1}) - \epsilon_{TG}(1715\ cm^{-1})/3$$

where FA, FAE and TG stand for fatty acid, fatty acid ester, and trigyceride, respectively.

If we apply the analysis outlined above to the reaction with the trioctanoylglycerol educt (Figure 3) with $\Delta\epsilon(1715\ cm^{-1})$ = 806 − 58/3 = 787 $M^{-1}\ cm^{-1}$, we obtain the following results (data not shown): after 90 min, for example, 53 mM octanoic acid is released, which of course corresponds to the same decrease in ester concentration. If we analyze the intensity decrease at 1161 cm^{-1} by using $\epsilon_{FAE}(1161\ cm^{-1}) \approx \epsilon_{TG}(1161\ cm^{-1})/3$ (in the case of trioctanoylglycerol: 772/3 = 257 $M^{-1}\ cm^{-1}$), we obtain a decrease in fatty acid ester concentration of 55 mM, compared with 53 mM from the analysis at 1715 cm^{-1}. The two results obtained from the analysis of these two separated regions in the spectrum are in good agreement.

Furthermore, formation of 53 mM octanoic acid after an incubation time of 90 min corresponds to a consumption of 53 mM water molecules during this time. This decrease in water concentration leads to a change in w_0 from $w_{0,st}$ = 11.1 to 10.0. If we would not have the contribution from the O–H (st) bands of the formed fatty acids and mono- and diglycerides (and eventually glycerol) in the water peak region, the decrease in optical density at 3457 cm^{-1} (l = 0.01 cm) would be 0.55; the observed change was 0.032.

In the following, all reactions are quantified by analyzing the infrared spectrum at 1715 cm^{-1} as outlined by using the experimentally determined molar extinction coefficients; see above.

Activity of R. delemar Lipase. In Figure 4A, the lipase-catalyzed hydrolysis of different triglycerides (C_6 to C_{10}) in 50 mM AOT/isooctane ($w_{0,st}$ = 11.1) is shown by using a constant initial triglyceride concentration of 50 mM. It can be seen that all four substrates are hydrolyzed and that after 2 h about 50–60 mM fatty acids are released in all cases,

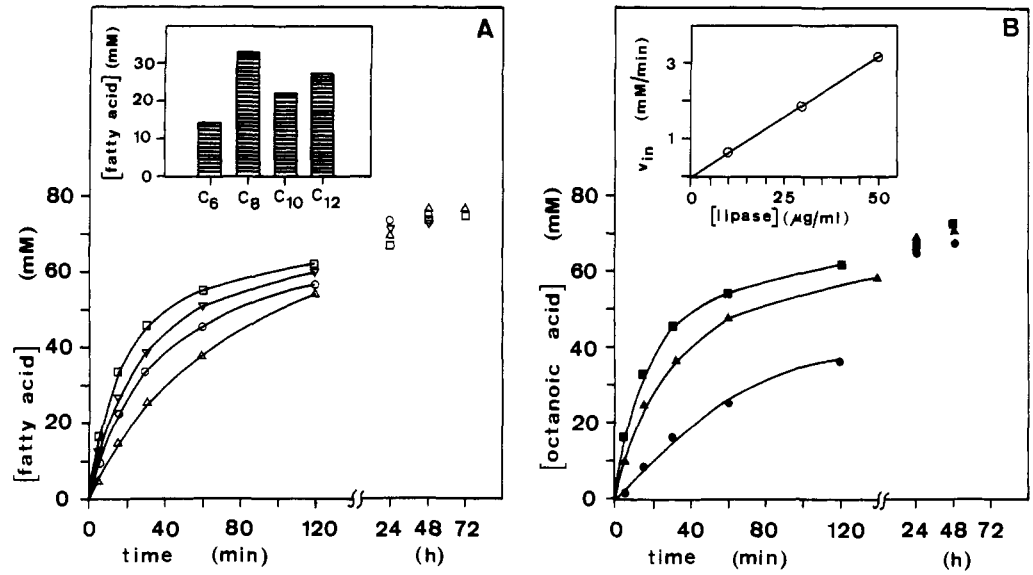

FIGURE 4: Activity of *R. delemar* lipase against different triglycerides in AOT reverse micelles. AOT (50 mM) in isooctane, $w_{0,st}$ = 11.1, $[triglyceride]_0$ = 50 mM, T = 25 °C. (A) Variation of the fatty acid chain length: (△) trihexanoylglycerol (C_6); (□) trioctanoylglycerol (C_8); (○) tridecanoylglycerol (C_{10}); (▽) tridodecanoylglycerol (C_{12}). Insert: Yield after 15 min. $[Lipase]_{0v}$ = 0.05 mg/mL. (B) Variation of the overall enzyme concentration: (●) 0.01 mg/mL; (▲) 0.03 mg/mL; (■) 0.05 mg/mL. Insert: Initial fatty acid production as a function of the overall lipase concentration.

corresponding to a yield of 50–60% [1,3 specificity of the enzyme assumed (Iwai & Tsujisaka, 1984)]; after 24 h, the yield was 60–70% (Figure 4A). Initially, trioctanoylglycerol is most rapidly hydrolyzed under the conditions used among the three triglycerides (insert of Figure 4A). Earlier studies using monoglycerides as substrates have also shown that the preferred fatty acid chain length for *R. delemar* lipase is eight carbon atoms (Iwai & Tsujisaka, 1984). Although a plateau is reached after 1 or 2 days of incubation, long-time measurements (over weeks) indicate that the true equilibrium of the reaction is not yet reached; slow hydrolysis of monoglycerides most probably occurs during longer incubation. However, we did not study this in more detail.

Using trioctanoylglycerol as substrate and varying the total enzyme concentration from 10 to 50 μg/mL (Figure 4B), we obtain a linear relation between the initial release of fatty acids and the enzyme concentration (insert of Figure 4B). Note that the yield after 24 h was independent of the enzyme concentration.

In Figure 5, the $w_{0,st}$ dependency of the trigyceride hydrolysis is shown for five initial w_0 values between 5.6 and 16.7. While the reaction yield after 2 or 24 h is almost the same for $w_{0,st}$ values above 8.3, a considerably lower value has been obtained for $w_{0,st}$ = 5.6, even after a longer incubation period. These observations most probably indicate a shift of the equilibrium constant (synthesis is getting more favorable). The effect of the initial water concentration on the equilibrium in lipase-catalyzed reactions in AOT reverse micelles has already been discussed in detail (Han et al., 1987b). In this context, it is worthwhile to mention that the concentrations of released fatty acids after a long-time incubation (>24 h) are independent of the amount of catalyst used at a given $w_{0,st}$ (Figure 4B). The amount of catalyst is only responsible for the speed by which the approach to the plateau value is reached.

In principle, the FTIR–reverse micelle–lipase assay is also applicable with naturally occurring triglycerides. The problem here arises from the fact that the extinction coefficient difference at 1715 cm^{-1} for the released fatty acids is not known exactly. We did not attempt to analyze the composition of the liberated fatty acid mixture; instead we assumed $\Delta\epsilon$(1715 cm^{-1}) to be 780 M^{-1} cm^{-1}, a mean value of the determined

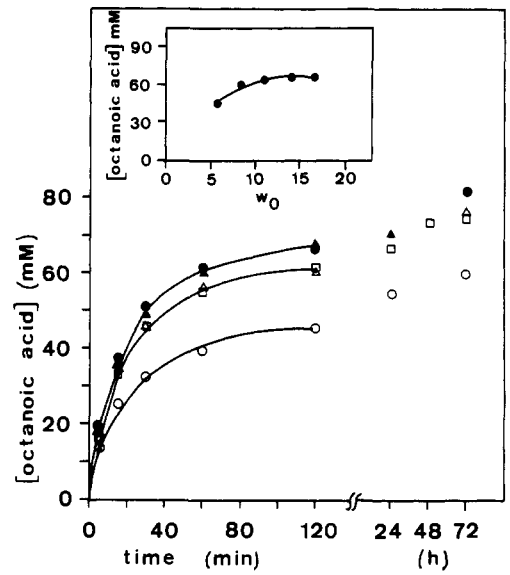

FIGURE 5: Activity of *R. delemar* lipase against trioctanoylglycerol in AOT reverse micelles. Variation of the total water content. AOT (50 mM) in isooctane, $[trioctanoylglycerol]_0$ = 50 mM, $[lipase]_{0v}$ = 0.05 mg/mL, T = 25 °C. $w_{0,st}$ = 5.6 (○), 8.3 (△), 11.1 (□), 13.9 (●), or 16.7 (▲). Insert: Yield after 120 min.

values for the C_6 to C_{12} acids.

We selected three vegetable oils that have different fatty acid compositions (Baumann et al., 1988): linseed oil (52% 18:3, 22% 18:1, 17% 18:2), sunflower oil (61% 18:2, 28% 18:1), and olive oil (64% 18:1, 16% 18:2, 14% 16:0). First of all, the maximum of the C=O (st) band lies in all three cases at 1751 cm^{-1}, typically for triglycerides (see above). Using an oil concentration of 7% (w/v), we obtained optical densities at 1751 cm^{-1} (l = 0.01 cm) of 0.95, 0.94, and 0.93 for linseed oil, sunflower oil, and olive oil, respectively. As seen in Figure 6A, the hydrolysis of all these vegetable oils is catalyzed by the lipase from *R. delemar* in AOT reverse micelles. If we use jojoba oil [which is not a triglyceride oil, but a mixture of linear fatty acid esters: 70% 20:1, 14% 22:1, 12% 18:1 (Baumann et al., 1988)], the reaction is comparatively slow, almost neglectible (Figure 6A). A distinction between the

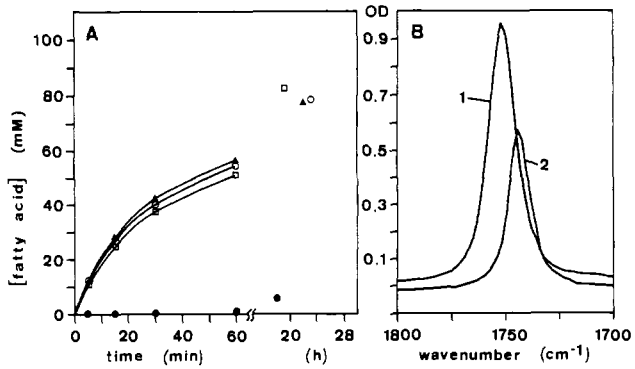

FIGURE 6: Vegetable oils as substrates for *R. delemar* lipase. (A) Activity of *R. delemar* lipase in AOT reverse micelles against linseed oil (▲), sunflower oil (○), olive oil (□), and jojoba oil (●). AOT (50 mM) in isooctane, $w_{0,st}$ = 11.1, [oil] = 7% (w/v), $[\text{lipase}]_{ov}$ = 0.05 mg/mL, T = 25 °C. (B) C=O (st) region of the FTIR spectrum of sunflower oil (1) and jojoba oil (2) in isooctane, [oil] = 7% (w/v).

two classes of oils can also be made on the basis of their C=O (st) peak maxima. In the case of jojoba oil, the peak maximum is at 1744 cm^{-1} (Figure 6B), comparable with ethyl esters from hexanoic, octanoic, decanoic, or dodecanoic acid; all show maximal (C=O (st) absorbance at 1745 cm^{-1}.

In order to check whether the activity of other lipases could also be studied in reverse micelles, we carried out a few measurements using 50 mM AOT in isooctane, $w_{0,st}$ = 11.1, and 50 mM trioctanoylglycerol as substrate. All the tested lipases were active: the lipases from *P. fluorescens*, *C. rugosa*, *A. niger*, and porcine pancreatic lipase.

Discussion

We have shown that FTIR spectroscopy can successfully be applied to the investigation of lipase activities in reverse micelles. The kinetics of the triglyceride hydrolysis can be followed with this method continuously with high reproducibility; it is not necessary to extract reaction products, and the quantitation occurs without chemical disturbance of the system.

Although the lipase-catalyzed hydrolysis of triglycerides is rather complex due to the formation of different reaction products (di- and monoglycerides, fatty acids, and eventually glycerol), the presence of isosbestic points in the spectrum simplifies the quantitative analysis remarkably. For this, one can select the C=O stretching band of the formed fatty acids at 1715 cm^{-1}, because changes here are comparatively large during the reaction and because standard curves can easily be made with commercially available fatty acids. It is therefore possible to determine the amount of fatty acids released during the reaction. In principle, one can carry out the analysis also in other regions of the spectrum, e.g., at 1161 cm^{-1} or at 1751 cm^{-1}; see Figure 3C,B.

Compared with other methods, in particular with the one that uses chromogenic substrates in the micromolar range (Fletcher et al., 1985; O'Connor et al., 1986), the FTIR method is of much lower sensitivity. We observe substrate conversions in the millimolar concentration range. However, in our case the substrates are triglycerides that are among the relevant ones in vivo. In addition, one could also use mono- or diglycerides as starting substrates or appropriate stereoisomers. This would in principle allow the determination of substrate specificity with respect to fatty acid position and stereospecificity which is frequently of greater interest (Jensen et al., 1983). In the present work, we limited ourselves mainly to triglyceride substrates.

Recognizing that an enzyme-containing micelle contains only a limited amount of water, one may be surprised by the relatively high extent of hydrolysis (i.e., water consumed). However, it has long been recognized (Fletcher & Robinson, 1981; Luisi et al., 1988) that among micelles there is a rapid exchange of materials. These exchange reactions are so fast that under normal conditions they are never rate limiting, i.e., they do not interfere with the slower chemical reaction. Under particular conditions, Han and Rhee (1985) have been observed that stirring somewhat increases the reaction velocity (most likely because it increases the exchange rate between the micelles). This effect has not been investigated in the present work.

As long as the catalyst can be solubilized in the reverse micelles and as long as the catalyst remains active, the FTIR method can in principle be applied for other reactions, involving water–insoluble substrates that can be dissolved in concentrations that are detectable with infrared spectroscopy (and/or with surfactant systems other than AOT).

Finally, an important application of FTIR spectroscopy in the field of reverse micelles in general is the determination of surfactant and/or water concentrations. In the latter case, at least as little as 0.1% (v/v) water can be determined with good accuracy.

The question of water is of course of central importance for enzymology at large and in the field of reverse micelles in particular. In fact, the physical properties of reverse micelles are strongly affected by water (Eicke, 1982; Luisi et al., 1988), and the study of the nature of water inside the water pool is of central interest for understanding the reactivity inside reverse micelles. The combination proposed in this paper between FTIR and reverse micelles, possibly further combined with circular dichroic measurements, appears therefore ideal to investigate lipases, and other enzymes, where small amounts of water may critically affect the activity. This study will be presented in a following paper.

Acknowledgments

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Registry No. C_6, 621-70-5; C_8, 538-23-8; C_{10}, 621-71-6; C_{12}, 538-24-9; lipase, 9001-62-1.

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Interactions of Antibody Aromatic Residues with a Peptide of Cholera Toxin Observed by Two-Dimensional Transferred Nuclear Overhauser Effect Difference Spectroscopy†

Jacob Anglister,* Rina Levy, and Tali Scherf
Department of Polymer Research, The Weizmann Institute of Science, Rehovot 76100, Israel
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ABSTRACT: The interactions between a peptide of cholera toxin and the aromatic amino acids of the TE33 antipeptide antibody, cross-reactive with the toxin, have been studied by NOESY difference spectroscopy. The 2D difference between the NOESY spectrum of the Fab with a 4-fold excess of the peptide and that of the peptide-saturated Fab reveals cross-peaks growing with excess of the peptide. These cross-peaks are due to magnetization transfer between the Fab and neighboring bound peptide protons, and a further transfer to the free peptide protons by exchange between bound and free peptide (transferred NOE). Additional cross-peaks appearing in the difference spectrum are due to a combination of intramolecular interactions between bound peptide protons and exchange between bound and free peptide. Assignment of cross-peaks is attained by specific deuteration of antibody aromatic amino acids using also the resonance assignment of the free peptide, deduced from the COSY spectrum of the peptide solution. The antibody combining site is found to be highly aromatic. We have identified one or two histidine, two tyrosine, and two tryptophan residues and one phenylalanine residue of the antibody interacting with valine-3, proline-4, glycine-5, glutamine-7, histidine-8, and aspartate-10 of the peptide. The 2D TRNOE difference spectroscopy can be used to study protein–ligand interactions, given that the ligand off rate is fast relative to the spin–lattice relaxation time of the protein and ligand protons (about 1 s). The resolution obtained in the difference spectra implies that the technique is equally applicable for studying proteins having a molecular weight larger than 50000.

Short synthetic peptides corresponding in sequence to segments of proteins are used to raise antipeptide antibodies cross-reactive with native antigens. Such peptides can be helpful in identifying pathogen-neutralizing epitopes and are potential synthetic vaccines (Arnon, 1986; Steward & Howard, 1987). Jacob and Arnon raised monoclonal antibodies against the synthetic peptide CTP3 (residues 50–64 of the B subunit of cholera toxin) that was previously suggested as a possible epitope for a synthetic vaccine against cholera (Jacob et al., 1983). The obtained antipeptide antibodies differ in their cross-reactivity with cholera toxin, implying that the same

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* Correspondence should be addressed to this author. He is the incumbent of the Graham and Rhona Beck Career Development Chair.

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