The Glycerol Ester Hydrolase (EC 3.1.1.3) from *Corynebacterium acnes*: a Serine Lipase†

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ABSTRACT: An extracellular lipase from *Corynebacterium acnes* has been partially purified and characterized. The enzyme was obtained from the crude culture broth after ultrafiltration, gel filtration through Sephadex G-100, and chromatography on CM-cellulose. This preparation demonstrates broad specificity and its activity against tri-, di-, and monoglycerides and also *p*-nitrophenyl acetate is completely inhibited after reaction with diisopropyl phosphorofluoridate (Dip-F). These substrates apparently react at the same catalytic site of the enzyme since: (1) a constant ratio of specific activities for the substrates *p*-nitrophenyl acetate and tributyrin is obtained during purification; (2) competitive inhibition can be demonstrated between these two substrates; (3) similar rates of decay of hydrolytic activity are observed with *p*-nitrophenyl acetate and triglycerides during either reaction with Dip-F or heat denaturation; and (4) reaction with [^{32}P]Dip-F yields only one [^{32}P]diisopropylphosphoryl (Dip) protein derivative with a molecular weight of approximately 54,000. This lipase is stable from pH 4–8 at 23°. It is relatively stable to heat at pH 5.5 but is heat labile at pH 7.8. [^{32}P]Dip-F was used to identify a reactive serine residue at the catalytic site. Presence of this serine residue suggests that the lipase contains a charge-transfer system similar to that described in such serine proteases as chymotrypsin, subtilisin, and elastase. Micellar particles of lipid substrates were analyzed for number, size, and stability with the Coulter counter, Model T. Following dilution of these particles into the concentration range required for K_M measurements, the micelles appear to dissolve. This physical instability of the substrate may explain the nonlinear enzyme kinetics often observed with the glycerol ester hydrolases.

The etiology of acne vulgaris, one of the most common of skin diseases, is complex (Fulton, 1972). As a consequence of androgenic stimulation of sebaceous glands at puberty, there is an associated increase in sebum flow (Strauss and Kligman, 1960). This sebum contains triglycerides which support the growth of an anaerobic gram-positive bacterium, *Corynebacterium acnes*, within the pilosebaceous follicles (Nicolaides and Wells, 1957; Kirshbaum and Kligman, 1963). An extracellular lipase from this organism hydrolyzes the triglycerides, providing glycerol for energy requirements. The other product of hydrolysis, the fatty acids, have been repeatedly incriminated as the irritants producing follicular plugging and, subsequently, acne vulgaris in genetically predisposed individuals (Strauss and Pochi, 1965; Kellum, 1968; Kligman *et al.*, 1970). In preparation for the development of specific enzyme inhibitors that might be used clinically, the following studies were carried out to purify and characterize this lipase. Previous studies have indicated that this lipase is recoverable from culture broth and demonstrates hydrolytic activity against purified lipid substrates (Reisner *et al.*, 1968; Freinkel and Shen, 1969; Fulton and Bradley, 1971; Hassing, 1971).

Experimental Section

Materials. Brain-Heart Infusion agar, Eugon broth, and casein were purchased from Difco Corporation. Tributyrin, trilaurin, triolein, tristearin, diolein, monolein, and [1-^{14}C]-trioctanoin (specific activity 84 Ci/mol) were obtained from Applied Science Labs. Sigma Chemical Co. supplied *o*-phospho-D,L-serine, diisopropyl phosphorofluoridate (Dip-F),[1] phenylmethylsulfonyl fluoride, bovine serum albumin, carbonic anhydrase, and lysozyme. Sephadex G-25, G-100, and G-200 were obtained from Pharmacia Fine Chemicals, and preswollen microgranular CM-cellulose (Whatman CM-52) was obtained from Reeve Angel Co. Bovine α-chymotrypsin, pancreatic lipase, salivary amylase, and glucose oxidase were purchased from Worthington Biochemical Corp. Subtilopeptidase A was the kind gift of Dr. Brian Catley. [^{32}P]-Dip-F was obtained from Amersham-Searle Corp.; the two lots used had specific activities of 76 and 80 Ci/mol. All other chemicals were of reagent grade.

Growth of Microorganisms. The bacterium, *Corynebacterium acnes*, was isolated from facial sebaceous follicles of 20 male and female volunteers without regard for a previous history of acne. All bacteria met the criteria for *Corynebacterium acnes, i.e.*, anaerobic gram-positive bacteria agglutinated by specific *C. acnes* antisera provided by the American Type Culture Collection. The bacteria were maintained as stock cultures on Brain-Heart Infusion agar (Zierdt *et al.*, 1968), and the three most active lipase producers were used as sources of enzyme. These were later classified as group II *C. acnes* or phage-resistant type (Voss, 1970). Three-day subcultures were inoculated into flasks containing 1 l. of Eugon broth enriched with 10 mM potassium phosphate buffer (pH 7.5), 55 mM glucose, and 0.08 mM sodium thioglycolate. To ensure anaerobiosis, the broth was gassed with nitrogen–

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[1] Abbreviations used are: NphOAc, *p*-nitrophenyl acetate; Dip-F, diisopropyl phosphorofluoridate; Cl-HgBzO, *p*-chloromercuribenzoate; Dip, diisopropylphosphoryl; $PhCH_2SO_2F$, phenylmethanesulfonyl fluoride.

carbon dioxide (95:5 v/v) for 15 min before inoculation. Cultures were maintained in a shaking metabolic incubator at 37° for periods up to 10 days. Glucose concentration in the media was determined by use of glucose oxidase (Pazar and Kleppe, 1964).

Purification Procedure. All purification procedures were conducted at 4°. Bacteria were removed from the broth by centrifugation at 22,500*g* for 60 min. The extracellular lipase from the broth was concentrated by ultrafiltration (XM-50 filter, Amicon Corp.). The retentate, concentrated to 10 ml, was percolated onto either a Sephadex G-100 or G-200 column (3.5 × 35 cm) and eluted with distilled water. The void volume, containing all of the enzymatic activity, was additionally purified by centrifugation (10,000*g* × 60 min) to sediment dense material, including melanoid pigments but not the lipase. The supernatant was lyophilized and reconstituted in 5 mM Tris-HCl (pH 7.2) for further purification by ion-exchange chromatography on a CM-cellulose column (2 × 20 cm). The final product was desalted by ultrafiltration, lyophilized, and stored at −20°.

Catalytic Activity. The broth was routinely assayed for lipase activity in the Metrohm pH-Stat using an olive oil-gum arabic emulsion in 0.1 mM NaCl–0.5 mM $CaCl_2$–4.5 mM sodium cholate solution (pH 7.8) (Shalita and Wheatley, 1970). Fatty acid release was monitored by potentiometric titration with 10 mM NaOH. After removal of the peptides from the bacterial broth by overnight dialysis against running tap water, protein concentration was determined using the Folin phenol reagent with bovine serum albumin as the standard (Lowry *et al.*, 1951). The specific activity of the enzyme preparation was expressed in units/mg of protein, one unit being equivalent to the titration of 1 nmol of NaOH/min.

Purified tributyrin, trilaurin, triolein, and tristearin were used for the kinetic studies following emulsification by sonication in Isoton (a particle-free saline solution provided by Coulter Electronics Inc., Hialeah, Fla.) containing sodium cholate (12.5 g/l.). The velocity of cleavage by the enzyme preparation was compared at concentrations between 0.125 and 5.0 mM. In view of the suggestions of relating enzyme kinetics with substrate surface area (Benzonana and Desnuelle, 1965), rather than with substrate concentration, attempts were made to measure particle size of the purified substrates at the concentrations tested on the Coulter counter (Model T, equipped with a 30-μ aperture tube) using latex particles as standards.

The activity of the lipase against NphOAc was determined in the NaCl–$CaCl_2$–sodium cholate solution (pH 7.8) described above. The extent of NphOAc hydrolysis was followed at 405 nm with a Cary 15 recording spectrophotometer, using a molar extinction coefficient of 1.84×10^4 (Behme and Cordes, 1967) to determine the nmoles of *p*-nitrophenolate generated/min.

The kinetic constants for NphOAc and tributyrin were evaluated by a double reciprocal plot (Lineweaver and Burk, 1934). Possible competitive substrate binding between these two substrates was studied by fixing the concentration of tributyrin at either 0.5 or 1.0 mM in the sample and reference cuvets of the Cary 15 spectrophotometer and following the enzymatic cleavage of increasing concentrations of NphOAc in the sample cuvet (0.05–1 mM).

To examine for the possibility of preferential hydrolysis of the α or β positions of the triglycerides by the bacterial lipase, an ethanolic solution of uniformly labeled [1-^{14}C]trioctanoin (0.6 μmol) was evaporated to dryness in the reaction vessel under a flow of nitrogen. The enzyme (240 units in 2 ml of Krebs-Ringer phosphate buffer (pH 7.5)) was added and the mixture sonicated to disperse the radioactive substrate (three 1-min bursts at 150 W with constant cooling in an acetone–Dry Ice bath; Sonifier Cell Disruptor, Model W 185 E, Heat Systems-Ultrasonics, Inc.). Incubations in a metabolic shaker at 37° were terminated after 15, 30, 60, or 120 min by the addition of chloroform–methanol (2:1 v/v). The lipids were extracted (Folch *et al.*, 1957) and streaked on silica gel plates for chromatography (Freeman and West, 1966). Radioactive products were located on the plates with a Vanguard Autoscanner, Model 818, and identified from standards similarly chromatographed (Fulton and Hsia, 1972). For comparison, simultaneous experiments, under identical conditions, were conducted with pancreatic lipase.

Proteolytic activity of the enzyme preparation was determined with casein as substrate (Awad *et al.*, 1972). These studies were conducted at 37° (pH 7.8) with incubations of 10, 20, 30, or 60 min.

Lipase activity of intact and sonicated saline-washed bacterial cells was measured with the olive oil emulsion. The sonicate was centrifuged (10,000*g* × 30 min) so that both the pellet and supernate of the sonicate could be examined for activity.

pH and Temperature Effects on Activity. The effect of pH on enzyme stability was studied by incubating samples of the enzyme for 30 min at 23° in water adjusted to pH values from 3 to 9 and determining the residual hydrolytic activity (pH 7.8, olive oil emulsion). The effect of pH on lipase activity against olive oil was determined by measuring initial enzyme velocities at pH values between 5.5 and 8.5.

The effect of temperature on stability was studied between 23 and 85° as follows: 0.1 ml of enzyme solution (pH 5.5) was diluted to 1 ml with either water or the olive oil emulsion at pH 5.5 and pH 7.8 and heated to the desired temperature for 15 min. Immediately thereafter it was plunged into ice for 30 min before assaying at 23° (pH 7.8, olive oil emulsion). For additional comparisons between substrates, the stability of the enzyme between 65 and 90° was again determined at pH 5.5 using either tributyrin or NphOAc as substrate.

Enzyme Inhibitors. Potential inhibitors were incubated with the enzyme at pH 7.6 for 120 min at 23° before residual enzyme activity was measured (pH 7.8, olive oil emulsion). Because of the remarkable inhibition observed with Dip-F, the rate of enzyme decay was examined after timed exposures to Dip-F of 10, 20, 30, 40, 60, or 120 min. The residual activity of the Dip-F treated enzyme was monitored with the following substrates: tributyrin, triolein, diolein, monolein, and NphOAc.

[^{32}P]Dip-F was used to label and identify the reactive residue at the catalytic site of the lipase (Awad *et al.*, 1972). Following partial acid hydrolysis of the [^{32}P]Dip-lipase, the mobilities of peptides containing ^{32}P were compared by high-voltage electrophoresis (Model HV 5000-S, Savant Instruments, Inc., Hicksville, N. Y.) at pH 1.9 and pH 3.5 to those of peptides similarly derived from [^{32}P]Dip-subtilisin and [^{32}P]Dip-chymotrypsin. The conditions for partial acid hydrolysis were modified by increasing the temperature from 105 to 130° to obtain a more complete digestion of the lipase derivative, and the buffer used at pH 1.9 was formic acid–acetic acid–water (2.5:8.7:88.8 v/v). The resultant electropherogram was exposed to X-ray film (GAF-X, General Aniline and Film Corp.) for 1 week for autoradiography.

Molecular Weight Determination. The molecular weight of the dissociated [^{32}P]Dip-lipase derivative was determined by

TABLE I: Purification Scheme of *C. acnes* Lipase.

Fraction	Total Protein[a] (mg)	Specific Activity[b] (units)	-Fold Purification	Yield (%)	NphOAc[c]/ Tributyrin ($\times 10^3$)
Culture supernatant	224[d]	650		100	5.8
Ultrafiltration (Diaflo, XM-50 filter)	52	2,660	4	95	6.0
Gel filtration (Sephadex G-100)	15	8,833	14	91	6.8
Centrifugation (10,000g $\times$ 60 min)	9	11,324	17	70	6.8
Chromatography (CM-cellulose)	3	24,752	38	51	7.1

[a] Total protein was determined by the method of Lowry *et al.* (1951). [b] Assay conditions were those described in the Experimental Section for tributyrin as substrate. [c] Ratio of specific activities for the hydrolysis of NphOAc and tributyrin. [d] Since 80% of the "protein" in the crude broth is diffusible through a cellulose membrane, the total protein was assayed following an overnight dialysis against running tap water.

sodium dodecyl sulfate polyacrylamide gel electrophoresis (Shapiro *et al.*, 1967; Weber and Osborn, 1969). Bovine serum albumin, salivary amylase, carbonic anhydrase, chymotrypsinogen A, and lysozyme were used as reference proteins. The gels with the [^{32}P]Dip-lipase derivative were cut longitudinally, using one-half for staining with Coomassie Blue and the other half for analysis of radioactivity. The latter half-gels were either: (1) dehydrated on filter paper on a Büchner funnel by rapid suction to prevent shrinkage and then exposed to X-ray film for autoradiography; or (2) sectioned horizontally into 1-mm sections for scintillation counting in a toluene–phosphor solution (Packard TriCarb liquid scintillation spectrometer, Model 2002). Using the standard semilogarithmic plot to determine approximate molecular weights, the mobility of the [^{32}P]Dip-lipase derivative was compared to the mobilities of the above reference proteins.

Results

Following inoculation of *C. acnes* into liquid broth, a rapid burst in bacterial replication preceded the production of lipase. Within the first 48 hr, maximum bacterial growth occurred but lipase activity was minimal in both bacteria and broth. Enzyme activity increased dramatically and linearly between the second and eighth day of incubation. This change from cell growth to enzyme production was associated with a pH shift from 7.5 to 5.5 and utilization of one-half of the glucose in the liquid broth.

The steps employed in the purification of the extracellular lipase are summarized in Table I. In the initial step, the enzyme was retained and concentrated by the XM-50 ultrafiltration membrane. The enzyme was completely excluded from Sephadex G-100 or G-200 and was significantly purified from low molecular weight material (Figure 1). Chromatography through DEAE-cellulose or hydroxylapatite resulted in a poor yield of enzymatic activity; whereas, 50% of the initial lipase activity was eluted from a CM-cellulose at moderate ionic strength (Figure 2). The modest amount of activity that escaped with buffer I in this preparation was the result of column overload, as on rechromatography of this fraction the activity was eluted with buffer III. Throughout the purification procedure, the ratio of specific activities of the enzyme against NphOAc and tributyrin remained within a narrow range (Table I).

The catalytic activity of the lipase against emulsions of triglycerides was linear for at least 30 min. Tributyrin demonstrated the highest V_{max}; triolein, trilaurin, and tristearin were cleaved at slower rates (Figure 3). When the kinetic data with tributyrin as substrate were analyzed by a double reciprocal plot, the results did not fall on a straight line (Figure 4). No improvement of the plot was noted after conversion of the data from substrate concentration to substrate surface area. One possible explanation for the failure to obtain a linear plot became apparent when the analysis of particle size of the lipid substrate in the Coulter counter revealed that the emulsion was unstable in the concentrations required for the kinetic measurements (Figure 5). Apparently the "velocity" measurements made at concentrations less

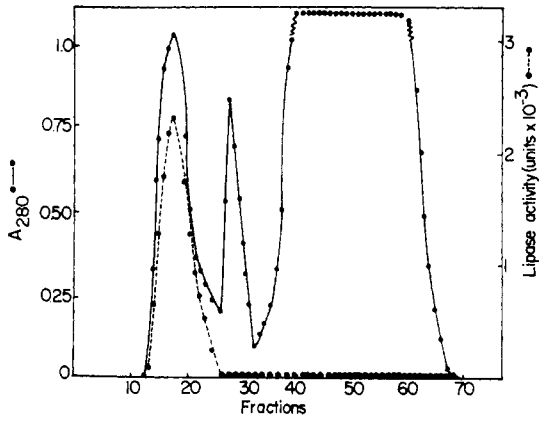

FIGURE 1: Sephadex G-100 filtration pattern. The enzyme was eluted from a 3.5 $\times$ 35 cm column with distilled water (pH 6.0) in 5-ml fractions at a flow rate of 30 ml/hr.

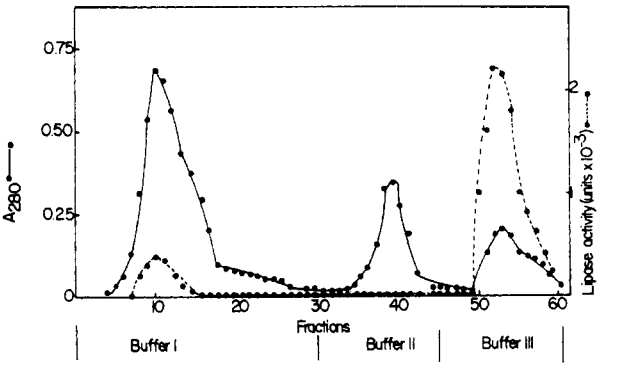

FIGURE 2: A chromatogram from CM-cellulose. Stepwise elution (2 $\times$ 20 cm column) was performed at pH 7.2 with 150 ml of buffer I (5 mM Tris-HCl), 75 ml of buffer II (50 mM Tris-HCl), and 75 ml of buffer III (50 mM Tris-HCl–0.5 M NaCl). Fractions of 5 ml were collected at a flow rate of 10 ml/hr.

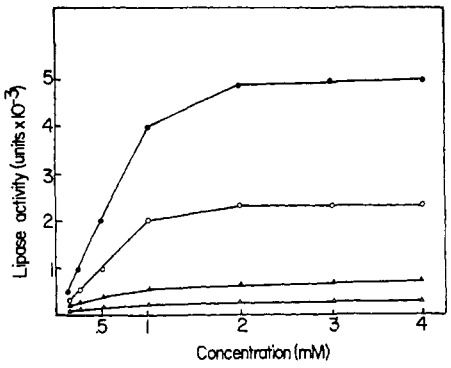

FIGURE 3: Rates of hydrolysis of tributyrin (●), triolein (○), trilaurin (▲), and tristearin (△) by the lipase of *C. acnes*. Emulsions of substrates were prepared with sodium cholate (12.5 g/l.) following the suggestions of Desnuelle. Cleavage was titrated potentiometrically with 0.01 N NaOH at 7.8 and 23° using the Metrohm pH-Stat.

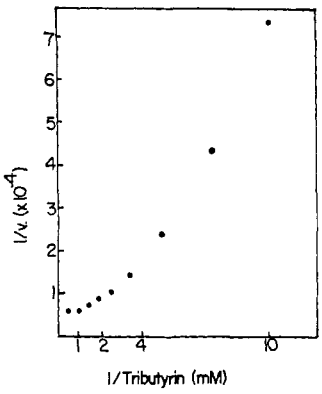

FIGURE 4: The double reciprocal plot of the hydrolysis of tributyrin by the lipase of *C. acnes*. Initial velocities were obtained by pH-Stat measurements using tributyrin emulsified with sodium cholate (12.5 g/l.).

than 1 mM reflect both enzymatic hydrolysis of the substrate and the removal of substrate by a rapid physical dissolution of the lipid particles into the aqueous phase.

Enzymatic hydrolysis of NphOAc was less rapid than that of tributyrin; yet, activity was sufficient for the determination of an apparent K_M of 3.3×10^{-4} M and a V_{max} of 4.25 units/mg. With the addition of enzyme to NphOAc, there was an immediate sharp increase in absorption at 405 nm; after 2 min, the reaction slowed and became linear for at least 10 min. This rapid burst in activity, followed by a leveling-off, suggests that deacylation, not acylation, is the rate-limiting step in catalysis (Behme and Cordes, 1967).

In the presence of saturating concentrations of tributyrin (1 mM), the catalytic activity of the lipase against NphOAc was totally inhibited. At a lower concentration of tributyrin (0.5 mM), the activity against NphOAc was diminished, but increasing concentrations of NphOAc could partially reverse this inhibition. These results with 0.5 mM tributyrin suggested a competition of the two substrates for the same catalytic site, and analysis of the kinetics confirmed this competitive inhibition, *i.e.*, an identical V_{max} and a shift in K_M from 3.3×10^{-4} to 10^{-3} M. Inactivation of the lipase preparations with Dip-F (Figure 6) or by heat (Table II) resulted in a similar decay of hydrolytic activity against both NphOAc and tributyrin, again indicative of a single catalytic site. Additionally, decay curves for the activity of Dip-F treated enzyme against triolein, diolein, and monolein were identical with that for tributyrin, providing further evidence that only one enzyme was involved in the hydrolysis of these several substrates.

TABLE II: Heat Inactivation of *C. acnes* Lipase as Monitored by Two Substrates.[a]

Temp (°C)	NphOAc		Tributyrin	
	Units	%	Units	%
65	2.01	100	481	100
70	1.43	71	348	72
75	1.20	60	220	45
80	0.84	42	128	26
85	0.52	26	72	15
90	0	0	0	0

[a] The enzyme solution (pH 5.5) was diluted to 1 ml in distilled water and heated to the indicated temperature for 15 min. Thereafter, the solution was plunged into an ice bath for 30 min in Methods before assaying residual activity at 23° and pH 7.8 using the tributyrin emulsion as substrate in the pH-Stat and NphOAc as substrate in the Cary 15 spectrophotometer. Results are expressed both in units and in % of initial velocity of a control preparation held at 23°.

Chromatographic analysis of the cleavage products of [1-^{14}C]trioctanoin after 15, 30, 60, or 120 min of hydrolysis indicated the absence of a monoglyceride intermediate at any time interval such as that found following the selective cleavage of triglyceride by pancreatic lipase (Figure 7). This suggests that this bacterial enzyme lacks substantial selectivity toward acyl sites on the triglyceride. In both the mammalian and microbial studies minimal amounts of diglycerides were detected. No proteolytic activity against casein could be demonstrated in the enzyme preparation.

Sonication of the intact bacterial cells increased the detectable cell-bound hydrolytic activity fivefold. However, no activity could be demonstrated in the supernate fraction of this sonicate. Preliminary attempts at solubilization of the activity from the cell pellet with solvents or detergents have been unsuccessful.

The enzyme was stable from pH 4 to 8 (Figure 8). Consequently, the enzyme was routinely stored at pH 5.0–5.5 at −20°. Under these conditions, enzyme activity remained unchanged for at least 6 months. Catalytic activity against olive oil was also pH dependent, with insignificant hydrolysis below pH 5.5. Optimal activity occurred between pH 7.6 and 8.0; accordingly routine assays were conducted at pH 7.8.

At pH 5.5, the enzyme was relatively stable to heat in either water or the olive oil emulsion, requiring 15 min at 75° to reduce the activity to less than 50%. At pH 7.8, the enzyme was more heat labile, losing 50% of activity after 15 min at 50° (Figure 9). The presence of the lipid substrate during this heating process had no appreciable effect on stability.

It was possible to inhibit the extracellular lipase with the two metals, copper and zinc; however, EDTA, Cl-HgBzO and $PhCH_2SO_2F$ were ineffective. Tetracycline-HCl was an inhibitor at high concentrations, while Dip-F was the most potent inhibitor, being effective at 10^{-6} M (Table III). Dip-F also inhibited the cell-bound lipase but to a lesser degree, producing 95% inhibition at 10^{-4} M and 37% inhibition at 10^{-5} M.

After partial acid hydrolysis of the [^{32}P]Dip-lipase, analysis of the migration of radioactive products at pH 3.5 (Figure 10) and pH 1.9 demonstrated conclusively that *o*-phosphoserine

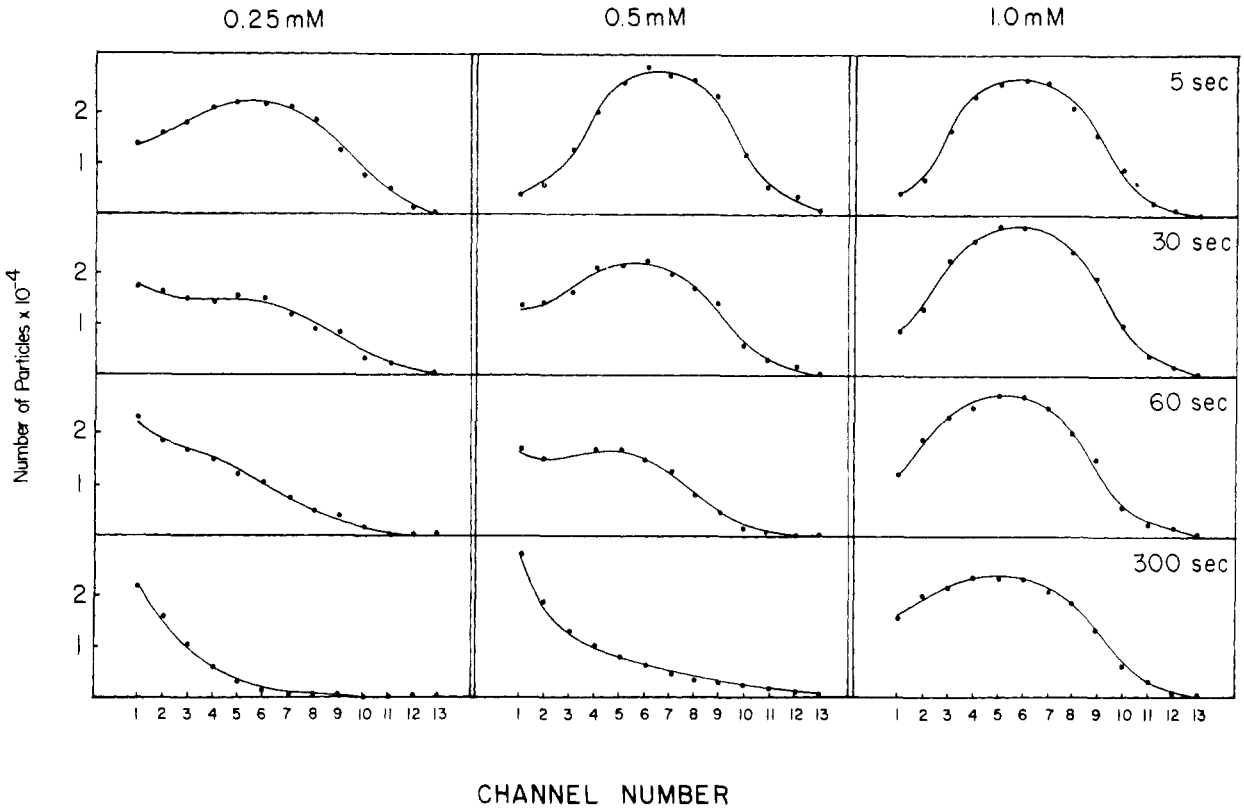

FIGURE 5: Distribution of particle sizes within emulsions of tributyrin (0.25, 0.5, and 1.0 mM). Measurements were conducted on the Coulter counter, Model T, utilizing 13 channels simultaneously. The sensitivity in each channel was adjusted so the smallest particle diameter detected from channel one to channel 13 was 0.63, 0.79, 1.00, 1.26, 1.59, 2.00, 2.52, 3.17, 4.00, 5.04, 6.35, 8.00, and 10.8 μ, respectively. Samples were rapidly diluted from a stock emulsion of triubtyrin (10 mM), allowing an initial reading at 5 sec. Monitoring was continued at 30-sec intervals for 10 min. The recordings at 5, 30, 60, and 300 sec are demonstrated.

was present, as in chymotrypsin and subtilisin. The band of greatest mobility is free [^{32}P]phosphate.

The sodium dodecyl sulfate polyacrylamide gel electrophoresis of the [^{32}P]Dip-lipase demonstrated a single radioactive band associated with a protein component (Figure 11). Analysis of the electrophoretic mobility of this [^{32}P]Dip-lipase derivative by the semilogarithmic plot was compatible with an approximate molecular weight of 54,000. It is apparent from these gels that the enzyme preparation either is not yet homogeneous or contains noncatalytic subunits of different molecular weights from a single enzyme.

Discussion

One of the significant findings in this study is the demonstration of a serine residue at the active site of the *C. acnes* lipase. Although similar studies in the past with a wide variety of other hydrolases have revealed a serine residue at the catalytic site, this is the first such example in the family of microbial glycerol ester hydrolases. Since serine is inherently

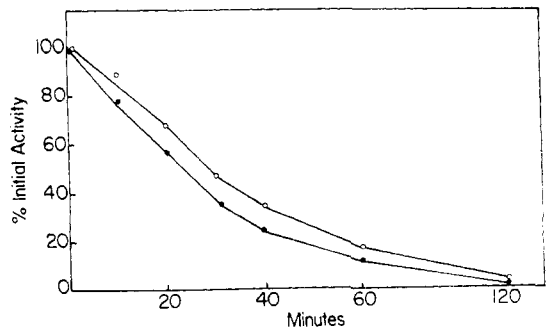

FIGURE 6: Loss of enzyme activity following the addition of Dip-F (0.1 mM). The hydrolysis of 2 mM tributyrin (●) was followed in the pH-Stat; the hydrolysis of 2 mM of NphOAc (○) was monitored at 405 nm in the Cary 15 spectrophotometer.

TABLE III: Effect of Enzyme Inhibitors on *C. acnes* Lipase.[a]

Inhibitor	Concn (mM)	Inhibn (%)
$Cu(CH_3COO)_2$	10.0	100
	1.0	87
	0.1	47
$ZnCl_2$	10.0	77
	1.0	27
	0.1	0
EDTA	10.0	32
	1.0	0
	0.1	0
Cl-HgBzO	1.0	0
$PhCH_2SO_2F$	1.0	0
Tetracycline-HCl	1.0	100
	0.1	41
Dip-F	0.1	100
	0.01	53
	0.001	34

[a] Inhibitors were preincubated with the enzyme preparation at pH 7.6 for 120 min at 23°. Residual activity was assayed on the Metrohm pH-Stat using the olive oil emulsion at pH 7.8. During incubation with EDTA, $CaCl_2$ was omitted from the salt solution.

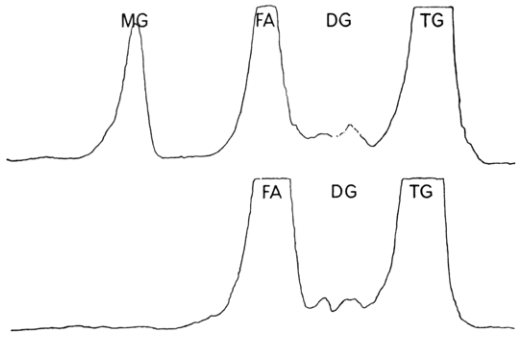

FIGURE 7: Tracings of radioactivity from thin-layer chromatograms of the 60-min hydroyltic products of [1-^{14}C]trioctanoin by pancreatic lipase (upper panel) and *C. acnes* lipase (lower panel): TG, triglycerides; DG, diglycerides; FA, fatty acids; MG, monoglycerides.

a poor nucleophile, it is inferred from these studies that a charge-transfer system is present in this enzyme, similar to that demonstrated among the specific serine, histidine, and aspartate residues in chymotrypsin (Sigler *et al.*, 1968; Blow *et al.*, 1969).

A recent report (Maylié *et al.*, 1972) described a similar specific serine residue at the catalytic site of pancreatic lipase. Since this latter enzyme is not effectively inhibited by Dip-F, an emulsion of diethyl *p*-nitrophenyl phosphate (4.5 mM) was required to demonstrate the serine residue in the sequence: -Leu-Ser-Gly-His-. Lipid-free preparations of the pancreatic lipase were necessary before the peptide digest could be separated by electrophoresis. We circumvented this problem by conducting the partial acid hydrolysis of the *C. acnes* lipase at an elevated temperature, yielding a more complete degradation of the enzyme and a better resolution during electrophoresis. In contrast to pancreatic lipase, several other lipases are similar to the one in the present study as these are inhibited by organophosphates presented to the enzyme in a soluble form and at relatively low concentrations (Rizack, 1960; Carter, 1967; Biale *et al.*, 1968; Somkuti *et al.*, 1969; Buchet and Lauwerys, 1970; Krysan and Guss, 1971).

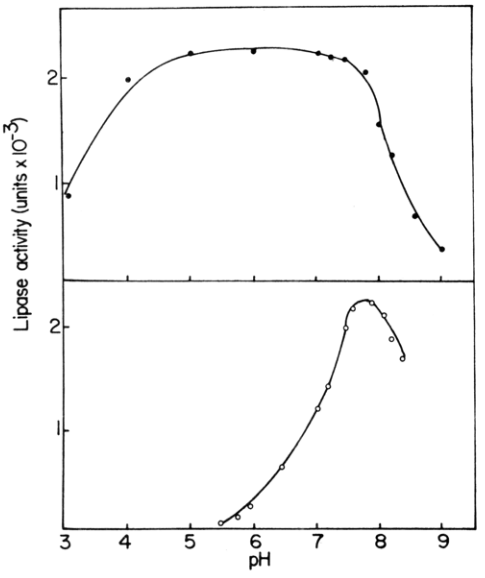

FIGURE 8: Effect of pH on enzyme stability (top panel) and enzyme activity (bottom panel). For the studies on enzyme stability, the samples of enzyme preparation were incubated at 23° in distilled water at pH values from 3 to 9 for 30 min prior to measuring the residual hydrolytic activity at pH 7.8 using the olive oil emulsion. The effect of pH on enzyme activity was monitored by measuring initial velocities at pH values between 5.5 and 8.5.

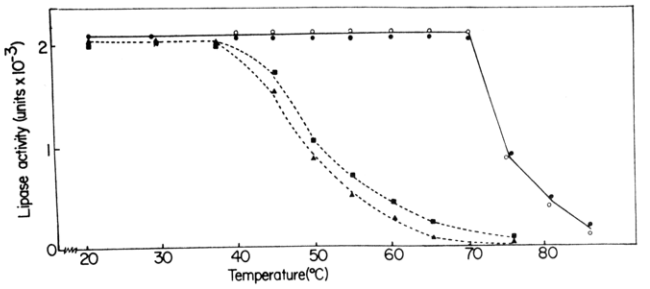

FIGURE 9: Effect of temperature on enzyme stability. The lipase preparation was heated for 15 min, then plunged into an ice bath for 30 min prior to measuring residual hydrolytic activity at 23° against the olive oil emulsion. The following conditions were examined: (●) pH 5.5, heated in distilled water; (○) pH 5.5, heated in an olive oil emulsion; (▲) pH 7.8, heated in distilled water; (■) pH 7.8, heated in an olive oil emulsion.

This microbial lipase also differs from pancreatic lipase as the enzyme apparently cleaves both soluble and insoluble substrates. The kinetic and denaturation studies with either NphOAc or tributyrin as substrate indicate a single catalytic site. This site also hydrolyzes diglycerides and monoglycerides as rapidly as triglycerides, thus explaining why these intermediates are not present during the hydrolysis of triglyceride. The finding of a single protein band labeled with ^{32}P after sodium dodecyl sulfate polyacrylamide gel electrophoresis of the [^{32}P]Dip-enzyme preparation confirms the interpretation of a single catalytic site with broad specificity. Other authors have also demonstrated that "esterase" and "lipase" activity by a single enzyme and have even suggested that "lipase" is a complex of "esterase" plus lipid (Okuda and Fujii, 1968).

The high molecular weight of the native form of the *C. acnes* lipase, as demonstrated by gel filtration, is not unusual and has been attributed in other lipases to aggregation of protein with lipid (Finkelstein *et al.*, 1970; Ramachandran *et al.*, 1970; Henderson, 1971). The dissociation of the native *C. acnes* lipase during sodium dodecyl sulfate polyacrylamide electrophoresis into a smaller component with a molecular weight of 54,000 may reflect the rupture of these protein–lipid interactions or the rupture of protein–protein bonds. The presence of three protein bands during sodium dodecyl sulfate polyacrylamide electrophoresis has been a constant finding throughout these studies despite the use of alternate purification methods, different strains of bacteria, or different culture broths. The two unlabeled protein bands may be either

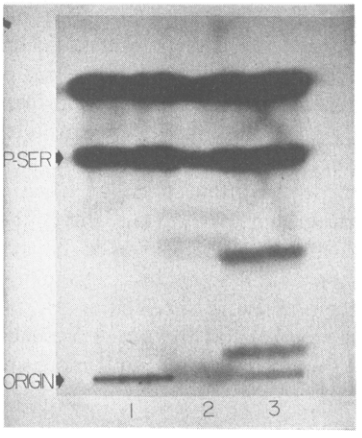

FIGURE 10: Autoradiogram of the partial acid hydrolysates of [^{32}P]Dip-chymotrypsin (band 1), the [^{32}P]Dip-lipase of *C. acnes* (band 2), and [^{32}P]Dip-subtilisin (band 3) following high-voltage electrophoresis (pH 3.5).

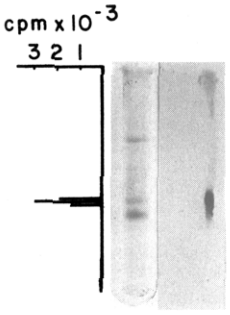

FIGURE 11: Demonstration of the [^{32}P]Dip-lipase derivative by scintillation counting (left panel) and by autoradiography on X-ray film (right panel) following sodium dodecyl sulfate polyacrylamide gel electrophosesis (middle panel).

contaminant proteins or components of a single macromolecular complex comprising the lipase of *C. acnes*.

Considerable controversy exists concerning the proper mode of expressing the Michaelis constants for lipases since substrates such as tributyrin do not form true solutions but are presented to the enzyme in micellar form. To circumvent this difficulty, Desnuelle and his colleagues (Sarda and Desnuelle, 1958) have advocated calculating the substrate concentration in terms of the total surface area of the stable emulsion. When we approached the measurements of surface area, using the 13-channel Coulter counter to evaluate simultaneously a range of particle sizes, it became apparent that dilution of the emulsion to the concentration required for our K_M determinations produced unstable emulsions. Consequently, kinetic constants obtained at low triglyceride concentrations represent the physical degeneration of the micelles as well as the hydrolysis of substrate. This problem of the kinetic analysis of insoluble substrates could be circumvented in our case since the *C. acnes* lipase has considerable activity against a soluble substrate. Using NphOAc, a conventional K_M of 3.3×10^{-4} M was calculated.

Analysis of stable emulsions (10 mM) on the Coulter counter did reveal differences in the mean particle sizes of the purified lipid substrates. Tributyrin and triolein droplets have mean diameters of 3 and 6 μ, respectively. Microscopic examination of emulsions of trilaurin and tristearin showed the presence of crystals instead of lipid droplets, thus, explaining their very low rate of hydrolysis.

Physical studies demonstrated a rapid rise in lipase activity between pH 5.5 and 7.8, suggesting the titration of a hydrogen ion on a histidine residue as in pancreatic lipase (Sémériva *et al.*, 1971). The loss of activity above pH 8 is apparently secondary to protein denaturation. This microbial lipase appears equally stable to heat whether dissolved in water or in an olive oil emulsion. In contrast, pancreatic lipase is more labile to heat denaturation when attached to lipid–water interfaces (Brockerhoff, 1969). Thus, the studies presented in this paper indicate that the enzymes in the class of glycerol-ester hydrolases are similar, yet diverse enough that generalizations about their absolute specificities for micelles as substrates or their physical stability in the presence or absence of these micelles should be limited until more studies are completed.

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Reconstitution of Transport Dependent on D-Lactate or Glycerol 3-Phosphate in Membrane Vesicles of *Escherichia coli* Deficient in the Corresponding Dehydrogenases†

Masamitsu Futai

ABSTRACT: By addition of purified D-lactate dehydrogenase to membrane vesicles made from an *Escherichia coli* mutant lacking this enzyme, it was possible to restore D-lactate dependent respiration and transport. This confirms the observations of J. P. Reeves, J.-S. Hong, and H. R. Kaback (1973, *Proc. Nat. Acad. Sci. U. S. 70*, 1917) made with a chaotropic extract of membranes from wild type cells as a source of enzyme. We have extended this procedure and obtained similar results on binding partially purified glycerol-3-phosphate dehydrogenase to membrane vesicles which were uninduced for this enzyme. In both systems, the greatest extent of reconstitution of respiration and stimulation of transport resulted from preincubation of deficient vesicles with dehydrogenase in 0.1 M potassium phosphate (pH 6.6) at 37° for 10 min. The nonpermeable electron acceptor, ferricyanide, completely inhibited the transport of amino acid in reconstituted vesicles driven by glycerol 3-phosphate. This would suggest that, as expected, the enzyme added back to vesicles remains on the outside. Unexpectedly, when vesicles made from cells in which the dehydrogenase had been induced in the normal fashion were examined, the uptake of amino acids driven by glycerol 3-phosphate was partially inhibited by ferricyanide. Transport by spheroplasts was not inhibited by this compound. We favor the interpretation that this dehydrogenase has moved outside during preparation of membranes. D-Lactate-driven transport by reconstituted vesicles was only slightly inhibited by ferricyanide, which was partly explained by the evidence that ferricyanide is a poor electron acceptor for purified D-lactate dehydrogenase.

The oxidation of D-lactate by the membrane bound D-lactate dehydrogenase greatly stimulates the active transport of various amino acids into membrane vesicles (see review by Kaback, 1972). An inducible dehydrogenase, glycerol-3-phosphate dehydrogenase, plays a similar role in membrane vesicles from cells induced for this enzyme (Dietz, 1971). Both enzymes have been solubilized from membranes and purified to apparent homogeneity (Kohn and Kaback, 1973; Futai, 1973; Weiner and Heppel, 1972).

Recently Reeves *et al.* (1973) made the striking observation that D-lactate dehydrogenase in guanidine-HCl extracts of membranes from wild type cells could restore D-lactate-dependent respiration and transport in membrane vesicles from mutants lacking this enzyme. This intriguing discovery encouraged us to attempt to determine if reconstitution was possible with highly purified enzyme and to see if other dehydrogenases are also active in restoration of respiration and transport. In the present investigation purified D-lactate dehydrogenase (Futai, 1973) or partially purified glycerol-3-phosphate dehydrogenase mixed with deficient membrane vesicles (Kaback, 1972) was found to function as a part of the oxidase system and was also observed to support the transport of amino acids.

Reeves *et al.* (1973) pointed out that reconstituted vesicles of this type differ from "natural" vesicles in their response to the D-lactate dehydrogenase inhibitor, oxamate. This may be due to the fact that the enzyme was bound to the outside of the vesicles, which may not be the normal site. In the present study, "natural" and reconstituted vesicles, both containing glycerol-3-phosphate dehydrogenase, were compared with respect to sensitivity to ferricyanide. This impermeant agent would act as a competing electron acceptor if it gained access to the dehydrogenase and therefore inhibit respiration and transport driven by glycerol 3-phosphate. The results suggested that glycerol-3-phosphate dehydrogenase in "natural" vesicles from normally induced cells had partly moved to the outside during preparation of membrane vesicles. By contrast, D-lactate-driven transport by reconstituted vesicles was only slightly inhibited by ferricyanide. Part of the reason for this is that ferricyanide is a poor electron acceptor for D-lactate dehydrogenase.

Materials and Methods

Bacteria and Growth Conditions. E. coli dld 3 (D-lactate dehydrogenase negative) derived from *E. coli* ML 308-225 ($i^-z^-y^+a^+$) was a generous gift from Dr. H. R. Kaback (Hong

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