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Lipoprotein Lipase Catalyzed Hydrolysis of Water-Soluble *p*-Nitrophenyl Esters. Inhibition by Apolipoprotein C-II†

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ABSTRACT: Bovine milk lipoprotein lipase (LpL) catalyzes the hydrolysis of the water-soluble esters *p*-nitrophenyl acetate (PNPA) and *p*-nitrophenyl butyrate (PNPB). The same protein and same active site are involved in hydrolysis of water-soluble *p*-nitrophenyl esters and emulsified trioleoylglycerol since (a) trioleoylglycerol hydrolysis and PNPB hydrolysis activities coelute from the heparin–Sepharose affinity column used to purify LpL and (b) LpL-catalyzed hydrolyses of trioleoylglycerol and PNPB are inhibited to equal extents by phenylmethanesulfonyl fluoride. The effect of apolipoprotein C-II (apoC-II) on the LpL-catalyzed hydrolysis of PNPA and PNPB has been determined. ApoC-II inhibits hydrolysis of both esters, with a maximum extent of inhibition of 70–90%. Inhibition of the LpL-catalyzed hydrolysis of PNPB is specific for apoC-II, since apolipoproteins A-I, C-I, and C-III-2 have little effect on this reaction, and is partial noncompetitive in form. K_I values for apoC-II inhibition of the LpL-catalyzed hydrolysis of PNPA and PNPB are in the range 0.26–0.83 μM. The effect of apoC-II on the temperature dependences of LpL-catalyzed hydrolysis of both esters and on NaCl inhibition of LpL-catalyzed PNPB hydrolysis is consistent with a change in rate-determining step when LpL and apoC-II interact. These results indicate not only that there is an interaction between apoC-II and LpL in aqueous solution in the absence of a lipid interface but also that this interaction conformationally modulates the active site of the enzyme.

Lipoprotein lipase (LpL)[1] is an enzyme that catalyzes the hydrolysis of triacylglycerols of chylomicrons and VLDL to produce 2-acylglycerols and fatty acids (Cryer, 1981, for review). LpL also catalyzes the hydrolysis of the *sn*-1 ester bond of phosphatidylcholine and phosphatidylethanolamine (Groot & Van Tol, 1978; Groot et al., 1978; Scow & Egelrud, 1976; Vogel et al., 1971). A protein constituent of VLDL and chylomicrons, apoC-II, is an activator of in vitro LpL-catalyzed hydrolysis of triacylglycerols in VLDL (Fitzharris et al., 1981; Matsuoka et al., 1981) and lipid emulsions (Krauss et al., 1973; Fielding, 1973; Ekman & Nilsson-Ehle, 1975; Schrecker & Greten, 1979; Bengtsson & Olivecrona, 1979; Havel et al., 1973; LaRosa et al., 1970) and of phosphatidylcholine in sonicated vesicles (Bengtsson & Olivecrona, 1980; Muntz et al., 1979; Stocks & Galton, 1980). In addition, severe hypertriglyceridemia in human subjects has been correlated with the absence of plasma apoC-II (Breckenridge et al., 1978; Cox et al., 1978; Yamamura et al., 1979). The molecular dynamic events of apoC-II activation of LpL catalysis are to date poorly defined.

Egelrud & Olivecrona (1973) have shown that bovine milk LpL catalyzes the hydrolysis of water-soluble esters such as PNPA. We have initiated a study of the purified bovine milk LpL-catalyzed hydrolysis of the water-soluble esters PNPA and PNPB. The use of water-soluble *p*-nitrophenyl esters as LpL substrates offers several experimental advantages. One is that at neutral and alkaline pH one of the products of LpL-catalyzed hydrolysis of *p*-nitrophenyl esters is *p*-nitrophenoxide, which strongly absorbs light at 400 nm, allowing for continuous monitoring of the reaction time course. Another advantage is that the kinetic parameters V_{max} and K_m are more easily interpreted for LpL-catalyzed hydrolysis of water-soluble

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[1] Abbreviations: apoA-I, apoC-I, apoC-II, and apoC-III-2, apolipoproteins A-I, C-I, C-II, and C-III-2, respectively; BSA, bovine serum albumin; DMF, dimethylformamide; DPPC, dipalmitoylphosphatidylcholine; LpL, lipoprotein lipase; PNPA, *p*-nitrophenyl acetate; PNPB, *p*-nitrophenyl butyrate; PNP, *p*-nitrophenoxide; PMSF, phenylmethanesulfonyl fluoride; HDL, high-density lipoproteins; VLDL, very low density lipoproteins; V_i, initial velocity; V_{max}, maximal velocity; K_m, Michaelis constant; k_{cat}, catalytic rate constant.

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substrates than for hydrolysis of triacylglycerols and phospholipids contained in organized structures such as lipoproteins and emulsions. This latter advantage accrues because LpL-catalyzed hydrolysis of lipid substrates in organized structures is a case of interfacial enzyme catalysis, for which K_m and V_{max} are only apparent values, as discussed by Verger & DeHaas (1976). Water-soluble *p*-nitrophenyl esters, then, promise to serve as convenient monitors of the molecular dynamics of LpL catalysis. In this report we show that the LpL-catalyzed hydrolyses of PNPB and emulsified trioleoylglycerol likely occur at the same active site. However, unlike LpL-catalyzed hydrolysis of lipid substrates which is activated by apoC-II, LpL-catalyzed hydrolyses of PNPA and PNPB are inhibited by apoC-II.

Materials and Methods

Preparation of LpL and Apolipoproteins. LpL was isolated from 8 L of bovine skimmed milk by affinity chromatography on heparin–Sepharose (Iverius, 1971), according to a modification (Matsuoka et al., 1980) of the procedure described by Kinnunen (1977). The purification was performed exactly as described previously (Matsuoka et al., 1980). LpL was eluted from the affinity column with 20 mM Tris-HCl, pH 7.4, containing 2 M NaCl. LpL activity was determined by using Triton X-100 emulsified trioleoylglycerol (Matsuoka et al., 1980) or PNPB (Shirai & Jackson, 1982) as substrate. Preparation of the trioleoylglycerol emulsion is described below (cf. PMSF Inhibition). Human apolipoproteins C-I, C-II, and C-III-2 (containing 2 mol of sialic acid/mol of apolipoprotein) were isolated from the VLDL of patients with type IV hyperlipoproteinemia, as previously described (Brown et al., 1969; Jackson et al., 1977). ApoA-I was isolated from human HDL (Baker et al., 1973).

Enzyme Kinetics. Time courses of the LpL-catalyzed hydrolysis of PNPA and PNPB were followed by monitoring production of *p*-nitrophenoxide (PNP) at 400 nm in 1 cm path length cells, using a Cary 219 UV–visible spectrophotometer. Reactions were monitored at pH 7.25 in a 0.1 M sodium phosphate buffer containing the indicated amounts of NaCl, LpL, heparin, and apolipoproteins and at the indicated temperatures. Temperature was controlled to ±0.1 °C by circulating water from a Lauda RM3 water bath through the cell holder of the spectrophotometer. Initial velocities (V_i's) were calculated from the slopes of the respective time courses over <10% reaction, using the equation

$$V_i\ [\mu\text{mol min}^{-1}\ (\text{mg of LpL})^{-1}] = 10^6 \frac{(\Delta A_{400}/\text{min})[[\text{assay volume (mL)}]/1000\ \text{mL}]}{\epsilon_{PNP}\ (\text{mg of LpL})} \quad (1)$$

where ΔA_{400}/min is the slope of the time course and ϵ_{PNP} is the absorptivity constant, in units absorbance M^{-1} cm^{-1}, determined from Beer–Lambert law plots of *p*-nitrophenol plus *p*-nitroxide at the experimental pH and ionic strength. In 0.1 M sodium phosphate buffer containing 0.1 M NaCl, pH 7.25, ϵ_{PNP} is 14 775; in the same buffer but containing 1 M NaCl, ϵ_{PNP} is 16 230. Background velocities for spontaneous hydrolysis of PNPA and PNPB were calculated as the slopes of the respective time courses prior to the addition of LpL. The exclusive contribution of LpL to the time course (ΔA_{400}/min in eq 1) was calculated by subtracting the slope of the background reaction from the slope measured after addition of LpL.

PMSF Inhibition. LpL (13.6 μg) was incubated with 1 mM PMSF in 1.09 mL of 0.1 M sodium phosphate, pH 7.25, containing 0.1 M NaCl, 18 μg of heparin, and 0.9% DMF for 50 min at 25.0 ± 0.1 °C, after which 10 μL of 0.01 M PNPB in acetonitrile was added and PNPB hydrolysis activity was measured by following the increase in absorbance at 400 nm; control experiments contained no PMSF. After PNPB hydrolysis activity was measured, 125-μL samples of each inhibition solution were removed and assayed in triplicate for the hydrolysis of trioleoylglycerol (Matsuoka et al., 1980). The trioleoylglycerol emulsion (125 μL) contained 4 mg/mL trioleoylglycerol, 0.4 μCi/mL tri[^{14}C]oleoylglycerol, 61 mg/mL BSA, and 0.06% w/v Triton X-100 in 0.3 M Tris-HCl buffer, pH 8.6. The enzyme solutions were incubated at 37 °C for 50 min, after which released [^{14}C]oleic acid was extracted by the method of Belfrage & Vaughan (1969) and radioactivity determined by liquid scintillation spectrometry. Controls contained all components save LpL.

Data Reduction Procedures. Initial velocity (V_i) vs. substrate concentration ([S]) data were fit to the Lineweaver–Burk transform of the Michaelis–Menten equation by weighted linear least-squares analysis, using a DEC-1160 minicomputer with RSX-11 operating system (CLINFO Computer Center, University of Cincinnati General Clinical Research Center) and PROGRAM ENZYME, written in Basic for this study. This program uses weighting factors that are directly proportional to V_i^4. The rationale for weighting linear least-squares fits to the Lineweaver–Burk equation has been presented by Cleland (1967). Weighted linear least-squares fits of data for apoC-II inhibition of LpL-catalyzed hydrolysis of PNPA and PNPB were also performed on the DEC-1160 minicomputer, using PROGRAM LINHIB, written for this study, and the mathematical approaches presented in the Appendix and under Results and Discussion.

Other Materials and Methods. Heparin, PMSF, BSA, DPPC, PNPA, and PNPB (Sigma Chemical Co., St. Louis, MO) were used as purchased. Tri[1-^{14}C]oleoylglycerol (50 mCi/mmol) was purchased from New England Nuclear. Both PNPA and PNPB released stoichiometric amounts of *p*-nitrophenol on hydrolysis at pH 13, based on absorbance at 400 nm and an absorptivity constant of 22 800. Stock solutions of PNPA and PNPB were prepared in acetonitrile which had been distilled from P_2O_5. DPPC vesicles were prepared by dissolving the lipid in chloroform, removing the chloroform by a stream of ultrapure nitrogen gas, and drying the resultant film in vacuo overnight. The lipid film was dispersed in the appropriate volume of 0.1 M sodium phosphate buffer, pH 7.25, and 0.1 M NaCl and sonicated for 30 min under a stream of nitrogen gas at ~50 °C on a Heat Systems Model W185F sonicator–cell disruptor equipped with a titanium microtip. Titanium particles were removed from vesicle preparations by centrifugation at maximum angular velocity in an IEC clinical centrifuge (Damon, IEC Division).

Results and Discussion

In order for water-soluble *p*-nitrophenyl esters to serve as suitable alternate substrates for the study of LpL catalysis, it must be established that their hydrolyses are catalyzed by the same protein and at the same active site as that for the hydrolysis of long-chain triacylglycerols, which are the preferred (natural) substrates of LpL. Support for the probable involvement of the same protein in trioleoylglycerol hydrolysis and PNPB hydrolysis comes from the data shown in Figure 1. Enzyme protein and hydrolysis activities toward trioleoylglycerol and PNPB coelute, suggesting that LpL is the likely catalyst of PNPB hydrolysis. Inhibition of the LpL-catalyzed hydrolysis of PNPB and of trioleoylglycerol by phenylmethanesulfonyl fluoride (PMSF) is shown in Table I. PMSF is an irreversible inhibitor of both LpL-catalyzed reactions.[2] LpL-catalyzed hydrolyses of PNPB and triol-

Table I: Inhibition of LpL-Catalyzed Hydrolyses of PNPB and Trioleoylglycerol by PMSF[a]

substrate	reaction velocity [μmol min^{-1} (mg of LpL)$^{-1}$]					
	−DPPC			+DPPC (0.6 mg/mL)		
	−PMSF	+PMSF	% inhibition	−PMSF	+PMSF	% inhibition
PNPB[b]	0.27 ± 0.01	0.17 ± 0.01	37 ± 4	1.47 ± 0.05	0.40 ± 0.03	73 ± 2
trioleoylglycerol[c]	1.55 ± 0.12	0.85 ± 0.12	45 ± 9	0.84 ± 0.15	0.26 ± 0.09	69 ± 12

[a] Procedures used to measure PMSF inhibition are described under Materials and Methods. [b] Velocities of PNPB hydrolysis are the means of two to four observations. Error limits are standard deviations of the mean. [c] Velocities of trioleoylglycerol hydrolysis are the means of six observations. Error limits are standard deviations of the mean.

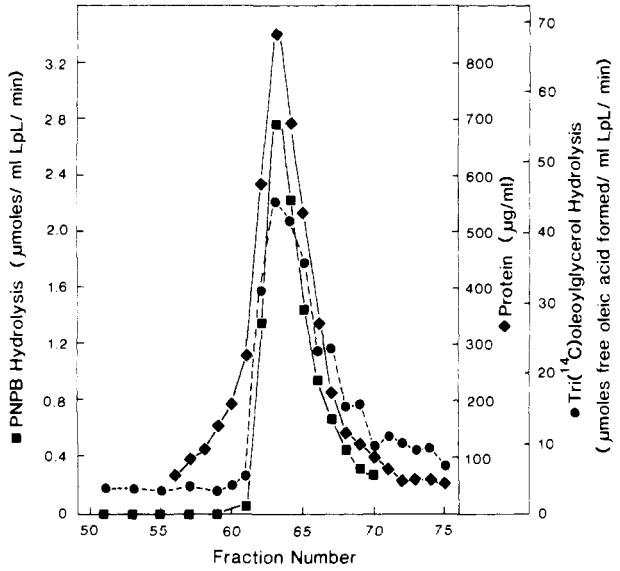


FIGURE 1: Chromatography of LpL on heparin–Sepharose. The enzyme was eluted from heparin–Sepharose by 2 M NaCl in 20 mM Tris-HCl buffer, pH 7.4, and 10-mL fractions were collected. An appropriate amount of each fraction was assayed for protein by the method of Lowry et al. (1951), for PNPB hydrolysis activity at 37 °C by using 0.5 mM PNPB in 1 mL of 0.1 M sodium phosphate, pH 7.2, containing 0.1 M NaCl, as described by Shirai & Jackson (1982), and for trioleoylglycerol hydrolysis activity at 37 °C (Matsuoka et al., 1980). For other details of the assay procedures, see Materials and Methods.

eoyltriglycerol are equally inhibited by PMSF either in the presence or in the absence of sonicated DPPC vesicles, indicating that the same active site is involved in both hydrolysis reactions. That a single active site on LpL is modified by PMSF is supported by the observation that the stoichiometry of labeling of LpL by [^{3}H]PMSF is ~1:1.[3] In addition, the velocities in Table I in the absence of PMSF yield a DPPC stimulation of 5.4-fold of the LpL-catalyzed hydrolysis of PNPB. This result agrees with the report by Shirai & Jackson (1982) that phospholipids stimulate LpL-catalyzed hydrolysis of PNPB.

Figure 2A shows representative time courses for the LpL-catalyzed hydrolyses of PNPA and PNPB; the velocities of both reactions depend linearly on the LpL concentration in the reaction mixtures (Figure 2B). The LpL-catalyzed hydrolysis of PNPA is slower than that of PNPB. This difference in activity is underscored by a comparison of the LpL kinetic parameters V_{max} and K_m, determined by Lineweaver–Burk linear least-squares analysis (plots not shown). V_{max} and K_m for PNPB hydrolysis are 6.73 μmol min^{-1} (mg of LpL)$^{-1}$ and 0.88 mM, respectively, at 25.0 ± 0.1 °C and pH 7.25. For

[2] D. M. Quinn, unpublished observations.
[3] L. S. Socorro, D. M. Quinn, and R. L. Jackson, unpublished observations.

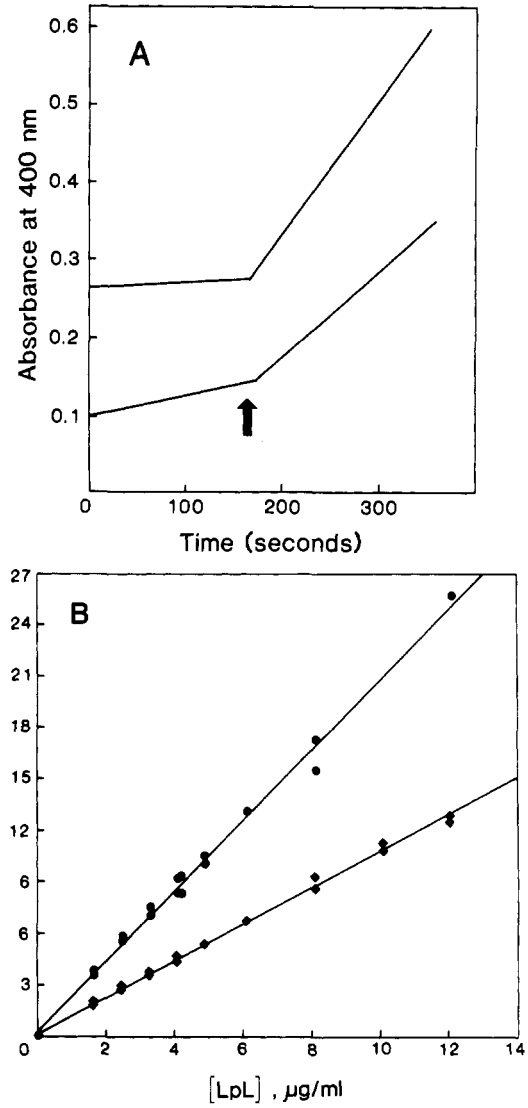


FIGURE 2: (A) Time courses for the LpL-catalyzed hydrolysis of PNPA (lower time course) and PNPB (upper time course). The reaction mixtures contained PNPB (0.5 mM) or PNPA (1 mM) in 0.1 M sodium phosphate, pH 7.25, containing 0.1 M NaCl and 5 μg/mL heparin at 25.0 ± 0.1 °C. The arrow is the point of injection of LpL to a final concentration of 4.1 μg/mL. The time course prior to LpL injection is that for background hydrolysis of substrates. (B) Dependence of the initial velocity on LpL concentration for the hydrolysis of PNPA (♦) and PNPB (●). Reaction conditions are identical with those described above.

PNPA hydrolysis under the same conditions V_{max} and K_m are 1.99 μmol min^{-1} (mg of LpL)$^{-1}$ and 1.03 mM, respectively. The fact that V_{max} is larger and K_m is smaller for the LpL-catalyzed hydrolysis of PNPB than for PNPA indicates that the enzyme has a greater specificity for the butyrate ester. Since the butyrate ester has a longer acyl chain than the acetate ester, these differences in kinetic parameters are

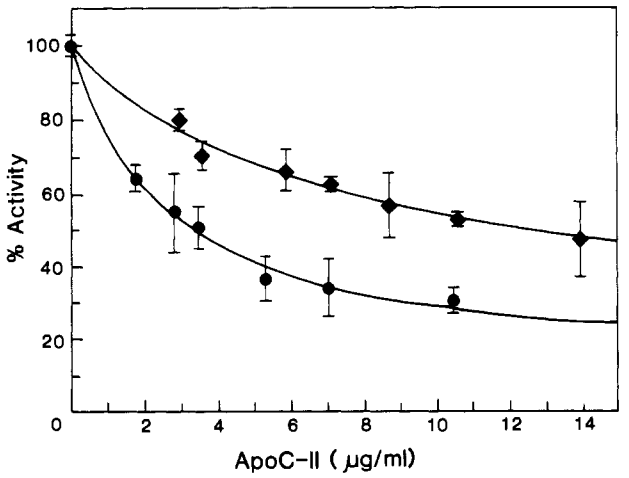


FIGURE 3: Inhibition of LpL-catalyzed hydrolysis of PNPA (●) and PNPB (♦) as a function of apoC-II concentration. Each reaction mixture contained 3.4 μg/mL LpL, 5 μg/mL heparin, and 1 mM PNPA or 0.5 mM PNPB. Each point is the mean of at least three determinations, and error limits are standard errors of the mean. The rates in the absence of apoC-II were 2.32 and 1.24 μmol min^{-1} (mg of LpL)$^{-1}$ for the LpL-catalyzed hydrolysis of PNPB and of PNPA, respectively. Other conditions are specified in the legend to Figure 2.

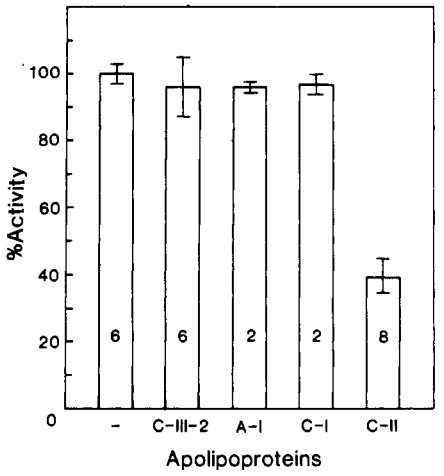


FIGURE 4: Effects of various apolipoproteins on the LpL-catalyzed hydrolysis of PNPB. Each reaction mixture contained 6.8 μg/mL LpL, 9 μg/mL heparin, 0.4 mM PNPB, and 9 μg/mL apolipoprotein. Numbers in each column represent the number of determinations. Error limits are standard errors of the mean, save when two determinations were done, for which error limits are the individual observations. The rate in the absence of apoproteins was 0.87 μmol min^{-1} (mg of LpL)$^{-1}$. Other conditions are specified in the legend to Figure 2.

consistent with the expected existence of a fatty acyl binding site contiguous with the esteratic locus (where ester bond breaking occurs) of the active site.

Even though PNPB and trioleoylglycerol hydrolyses appear to be catalyzed by the same LpL active site, the effect of apoC-II on the two reactions is different. It is well-known that apoC-II is an activator of the LpL-catalyzed hydrolysis of various water-insoluble lipid substrates (Fitzharris et al., 1981; Matsuoka et al., 1981; Krauss et al., 1973; Fielding, 1973; Schrecker & Greten, 1979; Ekman & Nilsson-Ehle, 1975; Bengtsson & Olivecrona, 1979; Havel et al., 1973; LaRosa et al., 1970; Stocks & Galton, 1980; Muntz et al., 1979). However, as Figure 3 illustrates, apoC-II inhibits the LpL-catalyzed hydrolysis of the water-soluble *p*-nitrophenyl esters, PNPA and PNPB. ApoC-II inhibition of the LpL-catalyzed hydrolysis of each ester is a saturable process, approaching

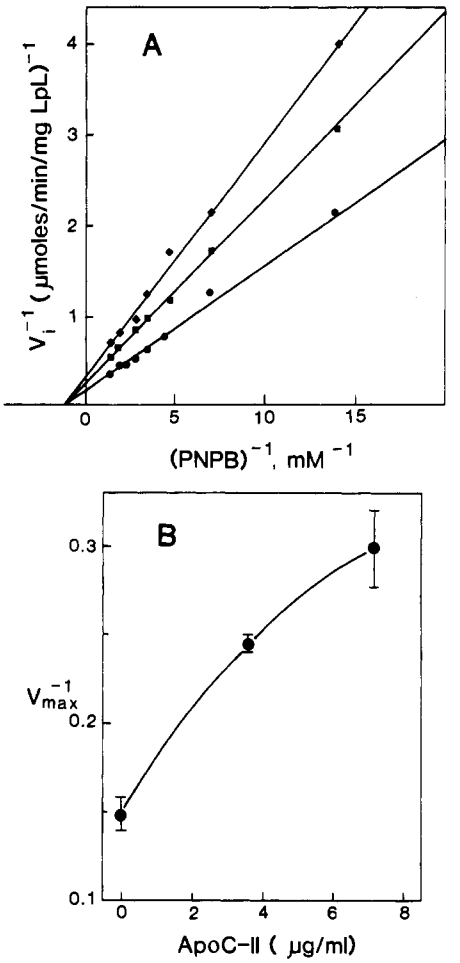
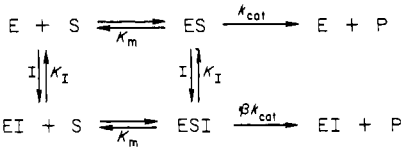


FIGURE 5: (A) Lineweaver–Burk pattern for inhibition of LpL-catalyzed hydrolysis of PNPB by apoC-II. ApoC-II concentrations were 0 (●), 3.6 (■), and 7.1 μg/mL (♦). Each point is the mean of at least three determinations. Other conditions are specified in the legends to Figures 2 and 4. (B) Replot of the intercepts of the Lineweaver–Burk plots of part A.

Scheme I



a maximum extent of inhibition of 70–90% at high apoC-II concentrations (i.e., apo-CII concentrations >30 μg/mL; data not shown). Therefore, apoC-II is a partial inhibitor of LpL-catalyzed hydrolysis of water-soluble *p*-nitrophenyl esters.

Since LpL is more specific for PNPB than for PNPA hydrolysis, apoC-II inhibition of the LpL-catalyzed hydrolysis of water-soluble *p*-nitrophenyl esters was further characterized by using PNPB. The data shown in Figure 4 indicate that only apoC-II inhibits LpL-catalyzed hydrolysis of PNPB. ApoA-I, apoC-I, and apoC-III-2 do not affect the reaction velocity to an extent greater than the experimental error.

The Lineweaver–Burk initial velocity pattern of Figure 5A shows that apoC-II is a noncompetitive inhibitor of the LpL-catalyzed hydrolysis of PNPB. Furthermore, since partial inhibition is observed, apoC-II inhibition is a case of simple, intersecting, hyperbolic noncompetitive inhibition (Segel, 1975). Two different preparations of LpL gave the same type of initial velocity pattern of PNPB hydrolysis. Figure 5B is a replot constructed from the *y*-axis intercepts of the Lineweaver–Burk weighted linear least-squares fits. The shape of

the replot is concave downward, consistent with hyperbolic inhibition of apoC-II.

The mechanism for simple, intersecting, hyperbolic noncompetitive inhibition of LpL-catalyzed hydrolysis of PNPB is shown in Scheme I. E, I, S, and P in Scheme I are LpL, apoC-II, PNPB, and *p*-nitrophenoxide plus *p*-nitrophenol, respectively. K_I is the dissociation constant of the EI and ESI complexes and β is the residual V_{max} at saturating apoC-II concentration. This scheme assumes that LpL and apoC-II form complexes of 1:1 stoichiometry. As we shall see below, a mathematical treatment using equations predicated on the mechanism of Scheme I yields reasonable fits to the data for apoC-II inhibition of the LpL-catalyzed hydrolyses of PNPA and PNPB. In addition, there is precedent in the literature for functionally important 1:1 complexes of LpL and apoC-II. Formation of 1:1 complexes of LpL and apoC-II has been suggested by Chung & Scanu (1977) for apoC-II enhancement of rat heart LpL-catalyzed hydrolysis of trioctanoin monolayers and by Fielding & Fielding (1977) for apoC-II enhancement of rat postheparin plasma LpL-catalyzed hydrolysis of lecithin-emulsified dioleoylglycerols. That K_I is equal for dissociation of EI and ESI in Scheme I is a sine qua non. If K_I were different in the two cases, the intersection of the Lineweaver–Burk plots would not fall on the $[S]^{-1}$ axis. However, the mean of the [S]-axis intercepts is 1.13 ± 0.075 mM^{-1}, illustrating the close agreement of the measured values. The dependence of initial velocity on PNPB and apoC-II concentrations is given by eq 2. From this equation it is apparent

$$V_i^I = \left(\frac{V_{max}[S]}{K_m + [S]}\right)\left(\frac{K_I + \beta[I]}{K_I + [I]}\right) \quad (2)$$

that both V_{max} ($[S] \gg K_m$) and V_{max}/K_m ($[S] \ll K_m$) have the same hyperbolic dependence on apoC-II concentration. Therefore, if the substrate concentration is held constant and the apoC-II concentration is varied, K_I and β can be determined. When [I] = 0, the initial velocity (V_i^0) is $V_{max}[S]/(K_m + [S])$, so that eq 2 can be transformed to

$$1 - \frac{V_i^I}{V_i^0} = \frac{[I](1-\beta)}{[I] + K_I} \quad (3)$$

The term on the left-hand side of eq 3 is the fraction of inhibition of LpL-catalyzed hydrolysis of PNPA or PNPB at any apoC-II concentration [I]. The double-reciprocal transform of eq 3 is linear

$$\left(1 - \frac{V_i^I}{V_i^0}\right)^{-1} = \frac{1}{1-\beta} + \frac{K_I}{1-\beta}\frac{1}{[I]} \quad (4)$$

and therefore β and K_I can be determined from the slope and intercept of the properly weighted linear least-squares fit. Derivation of the requisite weighting factors is presented in the Appendix.

Figure 6 shows the weighted linear least-squares fits to eq 4 of data of Figure 3 for the apoC-II inhibition of the LpL-catalyzed hydrolyses of PNPA and PNPB. The weighted linear least-squares fits are satisfactory and provide a computational verification of the mechanistic model outlined in Scheme I. The values of β and K_I calculated from the slopes and intercepts of the plots in Figure 6 are listed in Table II. The data of Table II were calculated from experiments by using two different preparations of LpL and illustrate the precision to which β and K_I are determined. The curvilinear fits of Figure 3 were generated from the β and K_I values calculated from the weighted linear least-squares fits of Figure 6. Since the properly weighted linear least-squares fit to eq 4 should give the same values for β and K_I as a nonlinear least-squares fit to eq 2, the curvilinear fits of Figure 3 also support the adequacy of the weighted linear least-squares computations detailed herein.

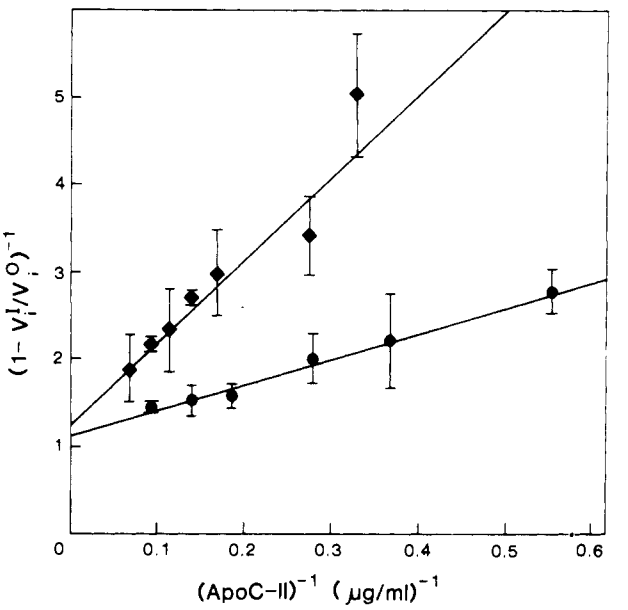


FIGURE 6: Weighted linear least-squares fits of the data of Figure 3, transformed according to eq 4 of the text, for the LpL-catalyzed hydrolysis of PNPB (♦) and PNPA (●).

Table II: Parameters for Inhibition of the LpL-Catalyzed Hydrolysis of Water-Soluble *p*-Nitrophenyl Esters by ApoC-II

substrate	β[a]	K_I (μM)[a]
PNPA	0.10 ± 0.02[b]	0.30 ± 0.01[b]
	0.33 ± 0.01	0.26 ± 0.01
PNPB	0.21 ± 0.03[b]	0.83 ± 0.05[b]
	0.30 ± 0.04	0.52 ± 0.05
	0.20 ± 0.05	0.49 ± 0.05

[a] Error limits are standard deviations calculated from the weighted linear least-squares fits. The individual β,K_I pairs were calculated from experiments with different preparations of LpL. [b] Values of β and K_I were calculated from the weighted linear least-squares fits shown in Figure 6.

The above analysis indicates that apoC-II is a partial noncompetitive inhibitor of the LpL-catalyzed hydrolyses of water-soluble *p*-nitrophenyl esters. The V_{max}, and hence k_{cat}, of the respective reactions is reduced by interaction of apoC-II and LpL. A germaine question is what the molecular basis of the observed inhibition by apoC-II is. The Eyring plots (Glasstone et al., 1941) in Figure 7, constructed from velocities measured at various temperatures in the range 12–37 °C, provide a clue to the answer. For both the LpL-catalyzed hydrolysis of PNPA and of PNPB the Eyring plots are nonlinear in the absence of apoC-II and can be described to a good approximation as intersecting straight-line segments. This computational interpretation is not exclusive; a curvilinear line can likely describe the data as well. However, the salient feature of the comparative Eyring plots for both esters is that the addition of apoC-II converts a biphasic plot into a linear (or more linear) plot. The downward breaks of the Eyring plots in the absence of apoC-II are experimental diagnostics of several possible molecular events (Dixon et al., 1979): (a) the stability of LpL over the time period required for assay varies with temperature: (b) a thermally induced change in rate-determining step occurs; (c) a temperature-dependent change in the structure of LpL and/or substrate occurs. The first possibility is not likely because preincubation of LpL for

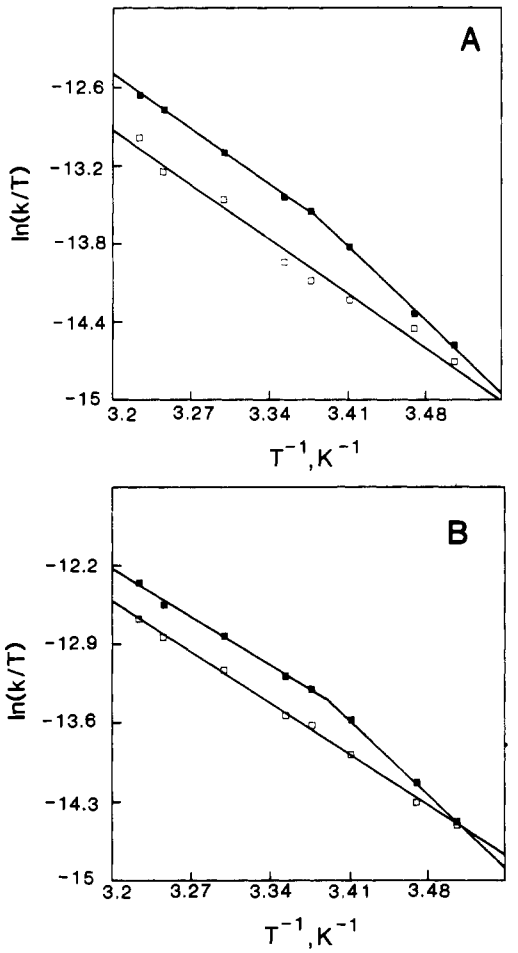


FIGURE 7: Eyring plots for the LpL-catalyzed hydrolysis of water-soluble *p*-nitrophenyl esters. (A) LpL-catalyzed hydrolysis of 1.9×10^{-4} M PNPA in the absence (■) and presence (□) of apoC-II (3.5 μg/mL). Each reaction mixture contained 8 μg/mL LpL and 10 μg/mL heparin in 0.1 M sodium phosphate, pH 7.25, containing 0.1 M NaCl. (B) LpL-catalyzed hydrolysis of 9.5×10^{-5} M PNPB in the absence (■) and presence (□) of apoC-II (6.9 μg/mL). Each reaction mixture contained 6.8 μg/mL LpL and 10 μg/mL heparin as in part A. Rate constants (k's) were the means of at least three determinations and were calculated by dividing initial velocities by initial substrate concentrations. The average precision of the means was ±4.9% and ±7% for LpL-catalyzed hydrolysis of PNPB and PNPA, respectively. Sufficiently low initial substrate concentrations were used such that $[S]_0 < 0.2K_m$, and therefore the calculated rate constant is $\sim V_{max}/K_m$.

2 min in buffer gives the same reaction velocity for PNPB within experimental error at 12, 25, and 37 °C as when there is no preincubation of LpL (data not shown). A temperature-dependent change in rate-determining step for the LpL-catalyzed hydrolysis of PNPB is supported by the NaCl inhibition data of Table III. At 12 °C, increasing the concentration of NaCl in the reaction medium from 0.09 to 0.9 M produces an inhibition of ~40%, whereas at 25 and 30 °C, the inhibition is ~20%. These differences in extent of inhibition suggest that different elementary steps, with distinct sensitivities to NaCl inhibition, contribute to the rate of reaction in the low- and high-temperature segments of the biphasic Eyring plots for the LpL-catalyzed hydrolysis of PNPA and PNPB (Figure 7). These suggestions do not preclude a thermally induced change in LpL conformation, since the reaction-dynamic manifestation of such a change could be a change in rate-determining step. Moreover, the fact that the Eyring plots for LpL-catalyzed hydrolysis of PNPA and PNPB break downward at about the same temperature (~25 °C) suggests that the break is due to the thermal sensitivity of

Table III: Effects of NaCl on the LpL-Catalyzed Hydrolysis of PNPB

temp (°C)	[apoC-II] (μg/mL)	$V_i^{0.9}/V_i^{0.09}$ [a]
12	0	0.63 ± 0.06
25	0	0.84 ± 0.04
	6.9	1.06 ± 0.09
30	0	0.80 ± 0.02
		0.77 ± 0.03 [b]
	8.5	1.1 ± 0.2
		1.0 ± 0.1 [b]

[a] [PNPB] = 1.9×10^{-4} M. Superscripts in the initial velocity ratio denote the molar concentration of NaCl in the reaction medium. Reactions were carried out in 0.1 M sodium phosphate buffers, pH 7.25. Each velocity was measured in triplicate. Error limits are standard errors, calculated from the fractional error: $\Delta R/R = [(\Delta V_i^{0.9}/V_i^{0.9})^2 + (\Delta V_i^{0.09}/V_i^{0.09})^2]^{1/2}$ where ΔV_i's are standard errors of the respective velocities and R is the initial velocity ratio. [b] [LpL] = 5.3 μg/mL. For all other experiments, [LpL] = 8.5 μg/mL.

Table IV: Activation Parameters for LpL-Catalyzed Hydrolysis of PNPA and PNPB

substrate	[apoC-II] (μg/mL)	$\Delta H^{\ddagger}$ (kcal mol^{-1}) [a]	$\Delta S^{\ddagger}$ (eu) [a]
PNPA	0	11.8 ± 0.3 [b]	−10.9 ± 0.8 [b]
		16.0 ± 0.6 [c]	3 ± 2 [c]
	3.5	11.8 ± 0.7	−11 ± 2
PNPB	0	12.1 ± 0.3 [b]	−9 ± 1 [b]
		18.5 ± 0.5 [c]	12 ± 2 [c]
	6.9	12.9 ± 0.2	−7.5 ± 0.7

[a] Error limits are standard deviations calculated from the linear least-squares fits. [b] Calculated from high-temperature segments of biphasic Eyring plots, corresponding to the temperature range 25–37 °C. [c] Calculated from low-temperature segments of biphasic Eyring plots, corresponding to the temperature range 12–25 °C.

LpL's conformation and not to temperature effects on substrate structure.

The conversion of the Eyring plots from biphasic to linear by the addition of apoC-II is accompanied by a change in the NaCl dependence of LpL-catalyzed hydrolysis of PNPB (cf. Table III); in the presence of apoC-II the reaction is insensitive to changes in the NaCl concentration from 0.09 to 0.9 M. These data suggest that interaction of apoC-II and LpL is accompanied by a conformational change in the LpL molecule at low temperature, where the slopes of the Eyring plots are different, and by increased resistance to NaCl-promoted loss of activity at high temperature, where the slopes of the Eyring plots are the same.

The suggestion that interaction of apo-CII and LpL is accompanied by a conformational change of the enzyme is implicit in the discussion of Bengtsson & Olivecrona (1979) on stimulation of LpL-catalyzed hydrolysis of 1(3)-monooleoylglycerol and trioleoylglycerol by apoC-II. The activation parameters for the LpL-catalyzed hydrolysis of PNPA and PNPB support this suggestion. The data in Table IV show that addition of apoC-II produces Eyring plots for the LpL-catalyzed hydrolysis of PNPA and PNPB that are characterized by $\Delta H^{\ddagger}$ and $\Delta S^{\ddagger}$ that are equal within experimental error (or nearly so) to those calculated from the high-temperature segments of the Eyring plots in the absence of apoC-II. The fact that the activation parameters from requisite Eyring plots are equal suggests that the same reaction step is rate controlling at all temperatures studied when apoC-II is present as when apoC-II is absent and the temperature is >25 °C. Hence, like increasing temperature in

the absence of apoC-II, interaction of LpL and apoC-II at low temperature appears to induce a change in the rate-determining step for LpL-catalyzed hydrolysis of water-soluble *p*-nitrophenyl esters. That such a change is mediated by conformational change of LpL when apoC-II and the enzyme interact is supported by the inhibition pattern of Figure 5. Since apoC-II is a partial noncompetitive inhibitor of LpL-catalyzed hydrolysis of PNPB, apoC-II does not prevent the binding of substrate to the active site, and inhibition occurs because catalytic turnover from the LpL–apoC-II–PNPB ternary complex is reduced relative to turnover from the LpL–PNPB binary complex. The simplest model that accounts for this inhibition behavior is that interaction of LpL and apoC-II results in change of the conformation of LpL's active site to one that is catalytically less potent for hydrolysis of water-soluble *p*-nitrophenyl esters.

Conclusion

The results in this paper show that LpL and apoC-II interact in aqueous solution in the absence of a lipid interface and that this interaction results in relay of conformational informatioin from the LpL–apoC-II binding site to the active site. Since the inhibition of the LpL-catalyzed hydrolysis of PNPB by apoC-II is well described computationally by expressions derived based on Scheme I, the stoichiometry of the LpL–apoC-II complex is 1:1, and the dissociation constant of the complex falls in the range 0.26–0.83 μM (cf. Table II). This range of dissociation constants compares favorably to the dissociation constant estimated by Bengtsson & Olivecrona (1979) of 0.34 μM from the amount of apoC-II required to produce half-maximal stimulation of bovine milk LpL-catalyzed hydrolysis of 1(3)-monooleoylglycerol dispersed with Triton X-100 and to the dissociation constants of Smith et al. (1982) for interaction of LpL and dansylated apoC-II fragments, which fall in the range 0.22–4.0 μM.

The mechanism described here for apoC-II inhibition of the LpL-catalyzed hydrolysis of water-soluble substrates may also operate when apoC-II stimulates the LpL-catalyzed hydrolysis of lipid substrates contained in organized structures such as lipoproteins. Such could occur if the conformation change that occurs when apoC-II and the enzyme interact produces an active site that is catalytically more potent for hydrolysis of lipid substrates (a V_{max} effect) and/or has a higher affinity for lipid substrate monomer (a K_m effect). Interestingly, the latter effect may apply to stimulation by apoC-II of LpL-catalyzed hydrolysis of emulsified trioleoylglycerol described by Schrecker & Greten (1979) and of triacylglycerols in VLDL described by Fitzharris et al. (1981) and Matsuoka et al. (1981), all of which are manifested by a large decrease in K_m with a small or no effect on V_{max}. Fielding (1973), on the other hand, showed that apo VLDL (which contains apoC-II) both increases V_{max} and decreases K_m of LpL-catalyzed hydrolysis of lecithin-emulsified triacylglycerols and diacylglycerols. Nonetheless, the fact that interaction of LpL and apoC-II can alternately inhibit hydrolysis of water-soluble *p*-nitrophenyl esters or stimulate hydrolysis of lipid substrates suggests that such interaction increases both the specificity and catalytic potency of LpL for lipid substrates.

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Appendix

Weighting of the linear least-squares fits to eq 4 is necessary because the native data set, initial velocity vs. apoC-II concentration, is functionally described by eq 2, of which eq 4 is a transform. Transformation not only renders the data in linear functional form but also produces a bias in the residuals of the linear least-squares fit that increases as the left-hand side of eq 4 increases (i.e., as the extent of inhibition decreases with decreasing apoC-II concentration). The weighting factors are derived by using eq 5 and 6:

$$F(V_i^0,V_i^I) = (1 - V_i^I/V_i^0)^{-1} \quad (5)$$

$$\mathrm{Var}[F(V_i^0,V_i^I)] = (\partial F/\partial V_i^0)^2\mathrm{Var}(V_i^0) + (\partial F/\partial V_i^I)^2\mathrm{Var}(V_i^I) \quad (6)$$

Equation 5 recognizes that eq 4 is a function of the dependent variables V_i^I and V_i^0. Equation 6 can then be used to obtain the variance of the transformed function (eq 4) from the variances in V_i^I and V_i^0. In eq 6, the partial derivatives are those of the function of eq 5 with respect to the dependent variables V_i^0 and V_i^I; $\mathrm{Var}(V_i^0)$ and $\mathrm{Var}(V_i^I)$ are the experimental variances of the respective dependent variables. The weighting factors for linear least-squares fit to eq 4 are determined by solving eq 6 and taking the reciprocal of the result:

$$W_i = \frac{[V_i^0(1 - V_i^I/V_i^0)]^4}{(V_i^0)^2 + (V_i^I)^2} \quad (7)$$

The variances of V_i^0 and V_i^I are assumed equal and hence do not appear in eq 7. A general discussion of the mathematical procedures used here to derive weighting factors for linear least-squares fits of enzyme kinetic data has been presented by Cleland (1967).

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Interaction of *Staphylococcus aureus* δ-Lysin with Phospholipid Monolayers†

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ABSTRACT: The interactions of purified staphylococcal δ-lysin and melittin with various phospholipid monolayers containing different polar head groups and fatty acid moieties and with monolayers of cod and sheep erythrocyte lipids at various initial film pressures (π_i) were studied by using the Wilhelmy plate method. In each case the final increase in surface pressure ($\Delta\pi$) was a linear function of π_i. In the case of δ-lysin, the critical pressures (π_c, the extrapolated values of π_i at $\Delta\pi$ = 0) for phosphatidylcholines with different fatty acid chain length, dipalmitoylphospholipids with different polar head groups, and cod or sheep erythrocyte total lipids fell within a relatively narrow range whereas melittin showed a much wider range. The collapse pressures of the δ-lysin and melittin films at the air–water interface when adsorbed from the hypophase were very similar. δ-Lysin showed little or no specificity in its interactions with all types of lipid films studied, whereas melittin showed preferential interaction with films of acidic lipid, similar to the specificity reported for cardiotoxins of *Naja mossambica mossambica* described by other workers.

δ-Lysin of *Staphylococcus aureus* is one of four principal extracellular proteins produced by numerous strains of the bacterium (Freer & Arbuthnott, 1976). It is a 26 amino acid polypeptide (Fitton et al., 1980) containing 14 hydrophobic residues and a high percentage of nonionizable side chain amino acids. Properties of the lysin such as solubility in chloroform–methanol (2:1 v/v), ethanol, and water, inactivation by phospholipids, and strong surface activity suggest an amphiphilic structure. It forms an unusually stable monolayer film at air–water interfaces similar to a lipid film, the collapse pressure depending on the solvent utilized (Colacicco et al., 1977).

Like δ-lysin, bee venom melittin, again a 26 amino acid amphiphilic polypeptide, displays strong surface activity and interacts with membranes and phospholipids. Melittin is predominantly hydrophobic in the first 20 residues (Habermann & Jentsch, 1967), but unlike δ-lysin the charge distribution is highly asymmetric with a cluster of four ionizable groups near the C terminus (residues 21–24, Lys-Arg-Lys-Arg) and single charges at the N terminus (Met) and lysine-7. Dawson et al. (1978) suggested a secondary structure for melittin consisting of two short α-helical sequences separated by a flexible "hinge" region centred on Pro-14. The basic C-terminal portion of the molecule is thought to interact with acidic groups on biomembranes, leading to planar orientation on the membrane surface. Subsequent conformational changes associated with hydrophobic interactions between the polypeptide and membrane lipids may result in a "wedgelike" insertion of melittin into the membrane and subsequent membrane destabilization. Fitton (1981) has suggested by the predictive method of Chou & Fasman (1974) that δ-lysin has a secondary structure very similar to that of melittin, with two α-helical domains separated by a flexible hinge region. A considerable α-helical content in aqueous solution is evident from the circular dichroism studies of Colacicco et al. (1977) and Fitton (1981). Partly on the basis of these similarities, a common mechanism for membrane penetration has been

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