Mushayakarara, E., & Levin, I. W. (1984) *Biochim. Biophys. Acta 769*, 585–595.
Papahadjopoulos, D., Vail, W. J., & Moscarello, M. A. (1975a) *J. Membr. Biol. 22*, 143–164.
Papahadjopoulos, D., Moscarello, M. A., Eylar, E. H., & Isac, T. (1975b) *Biochim. Biophys. Acta 401*, 317–335.
Pink, D. A., Chapman, D., Laidlaw, D. J., & Wiedmer, T. (1984) *Biochemistry 23*, 4051–4058.
Prendergast, F. G., Lu, J., Wei, G. J., & Bloomfield, V. A. (1982) *Biochemistry 21*, 6963–6971.
Schreier-Mucillo, S., Marsh, D., & Smith, I. C. P. (1976) *Arch. Biochem. Biophys. 172*, 1–11.
Sixl, F., & Galla, H.-J. (1982) *Biochim. Biophys. Acta 693*, 466–478.
Surewicz, W. K., & Epand, R. M. (1984) *Biochemistry 23*, 6072–6077.
Uemura, A., Kimura, S., & Imanishi, Y. (1983) *Biochim. Biophys. Acta 729*, 28–34.
Van Zoelen, E. J. J., Van Dijck, P. W. M., De Kruijff, B., Verkleij, A. J., & Van Deenen, L. L. M. (1978) *Biochim. Biophys. Acta 514*, 9–24.
Wiener, J. R., Pal, R., Barenholz, Y., & Wagner, R. R. (1983) *Biochemistry 22*, 2162–2170.

Solvent Isotope Effects for Lipoprotein Lipase Catalyzed Hydrolysis of Water-Soluble *p*-Nitrophenyl Esters†

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ABSTRACT: Solvent deuterium isotope effects on the rates of lipoprotein lipase (LpL) catalyzed hydrolysis of the water-soluble esters *p*-nitrophenyl acetate (PNPA) and *p*-nitrophenyl butyrate (PNPB) have been measured and fall in the range 1.5–2.2. The isotope effects are independent of substrate concentration, LpL stability, and reaction temperature and hence are effects on chemical catalysis and not due to a medium effect of D_2O on LpL stability and/or conformation. pL (L = H or D) vs. rate profiles for the V_{max}/K_m of LpL-catalyzed hydrolysis of PNPB increase sigmoidally with increasing pL. Least-squares analysis of the profiles gives $pK_a^{H_2O} = 7.10 \pm 0.01$, $pK_a^{D_2O} = 7.795 \pm 0.007$, and a solvent isotope effect on limiting velocity at high pL of 1.97 ± 0.03. Because the pL–rate profiles are for the V_{max}/K_m of hydrolysis of a water-soluble substrate, the measured pK_a's are intrinsic acid–base ionization constants for a catalytically involved LpL active-site amino acid side chain. Benzeneboronic acid, a potent inhibitor of LpL-catalyzed hydrolysis of triacylglycerols [Vainio, P., Virtanen, J. A., & Kinnunen, P. K. J. (1982) *Biochim. Biophys. Acta 711*, 386–390], inhibits LpL-catalyzed hydrolysis of PNPB, with $K_i = 6.9$ μM at pH 7.36, 25 °C. This result and the solvent isotope effects for LpL-catalyzed hydrolysis of water-soluble esters are interpreted in terms of a proton transfer mechanism that is similar in many respects to that of the serine proteases.

Lipoprotein lipase (LpL)[1] plays a central role in cardiovascular lipid metabolism [see Quinn et al. (1983) for a review]. Its primary physiological task is the hydrolytic cleavage of the *sn*-1 and *sn*-3 ester bonds of triacylglycerols that are transported in the bloodstream in VLDL and chylomicrons. The catabolic products of LpL catalysis, IDL and chylomicron remnants, are thought if cholesteryl ester rich to be particularly atherogenic (Zilversmit, 1977, 1979). Lack of plasma LpL activity (Havel & Gordon, 1960) or of the LpL activator apoC-II (Breckenridge et al., 1978; Cox et al., 1978) is correlated with clinical manifestations of serum hypertriacylglycerolemia. It has been suggested that chylomicron and VLDL surface elements that are released from the catabolic lipolysis complex are nascent HDL (Tall & Small, 1980). The case for an antiatherogenic role for HDL appears to be gaining momentum with the passage of time (Kannel et al., 1979; Miller, 1980).

Despite the great physiological significance of LpL activity, there are glaring inadequacies in our knowledge of the enzyme's catalysis. This dearth of information obtains from the fact that the details of the chemical steps of LpL action have not been defined, which in turn hampers efforts to build a thoroughgoing biodynamical model of such molecular events as activation of LpL by interaction with lipid interfaces (Shirai & Jackson, 1982) or with apoC-II [see Quinn et al. (1983) and references cited therein]. Furthermore, efforts to design synthetic effectors of LpL catalysis, whether they be inhibitors or activators, cannot proceed until chemical catalysis by the enzyme is understood.

This paper represents the initiation of efforts to expose the chemical strategies employed by LpL to achieve its catalytic aim. Solvent deuterium isotope effects for the LpL-catalyzed hydrolysis of the water-soluble substrates *p*-nitrophenyl acetate (PNPA) and *p*-nitrophenyl butyrate (PNPB) have been measured and delineated as specific effects on the rates of chemical steps of catalysis, rather than as effects of D_2O on

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[1] Abbreviations: LpL, lipoprotein lipase; IDL, intermediate-density lipoproteins; HDL, high-density lipoproteins; VLDL, very low density lipoproteins; PNPA, *p*-nitrophenyl acetate; PNPB, *p*-nitrophenyl butyrate; V_i, initial velocity; V_{max}, maximal velocity; K_m, Michaelis constant; apoC-II, apolipoprotein C-II; PMSF, phenylmethanesulfonyl fluoride; K_i, competitive inhibitor dissociation constant; pL, pH or pD; PNP, *p*-nitrophenoxide; Tris, tris(hydroxymethyl)aminomethane.

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enzyme stability or gross conformation. The pK_a of active-site groups involved in catalysis has been determined from pH–rate profiles and is not subject to the ambiguities that plague pH effects on the rates of lipolytic enzyme-catalyzed heterogeneous reactions. The results described herein allow postulation of an LpL catalytic mechanism and mode of transition-state stabilization that is similar to that of serine proteases (Blow, 1976; Hegazi et al., 1978) and acetylcholinesterase (Rosenberry, 1975a).

Materials and Methods

Materials. LpL was purified from 30 L of skimmed bovine milk as described previously (Kinnunen, 1977; Matsuoka et al., 1980). PNPA, PNPB, benzeneboronic acid, and the sodium salt of heparin from porcine intestinal mucosa were obtained from Sigma Chemical Co. and were used as received. Both PNPA and PNPB released stoichiometric amounts of *p*-nitrophenoxide on total hydrolysis at pH 13 and based on an absorptivity constant at 400 nm of 21 390 M^{-1} cm^{-1}. D_2O was purchased from Aldrich Chemical Co. and was used as delivered. All other materials were commercially available reagent-grade products.

Enzyme Kinetics. Initial velocities of LpL-catalyzed hydrolysis of PNPA and PNPB were calculated as slopes of initial portions of time courses constructed by following *p*-nitrophenoxide production at 400 nm with a Beckman DU7 UV–visible spectrophotometer. Initial velocities for the background hydrolysis of substrates were determined in the absence of LpL and subtracted from velocities determined in the presence of enzyme. Time course data (digital absorbance readings vs. time) were accumulated by using an IBM Personal Computer interfaced to the spectrophotometer, and slopes were calculated by linear least-squares analysis, using programs written in BASIC by the author. Temperature was controlled (±0.1 °C) by circulating water from a Lauda RC3 water bath through the spectrophotometer cell holder. Reaction velocities in units of molar per second were calculated from slopes of time courses (dA/dt, units of absorbance per second) according to the equation:

$$V_i = -d[S]/dt = \frac{dA/dt}{\epsilon_{PNP}} \quad (1)$$

The absorptivity constant ϵ_{PNP} is that for *p*-nitrophenoxide at 400 nm and is corrected for the fraction of total *p*-nitrophenol product in the *p*-nitrophenoxide form at the indicated experimental pL (L = H or D) and ionic strength, using pK_a's of *p*-nitrophenol measured by spectrophotometric titration of 6.98 and 7.48 in H_2O and D_2O, respectively (data not shown). Buffer pHs were measured by using a Corning Model 125 pH meter and glass combination electrode; pDs of buffered D_2O solutions were calculated by adding 0.40 to the pH meter reading (Salomaa et al., 1964).

Data Reduction Procedures. All fits of experimental data shown in various figures of this manuscript were calculated by least-squares procedures on the IBM Personal Computer alluded to above by using programs written by the author. Linear fits to the Lineweaver–Burk transform of the Michaelis–Menten equation were calculated by weighted least squares, using weighting factors proportional to V_i^4, as outlined by Cleland (1967). Nonlinear fits of pL–rate profiles (L = H or D) were calculated by using eq 2, which describes LpL activity as depending on the basic form of a single titrable amino acid side chain:

$$V_i = \frac{V_{i,lim}K_a}{[H^+] + K_a} \quad (2)$$

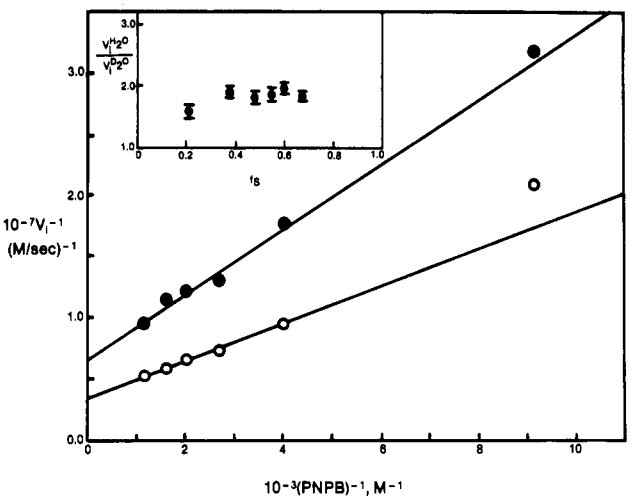


FIGURE 1: Lineweaver–Burk plots for LpL-catalyzed hydrolysis of PNPB. Each point is the mean of at least two determinations. Runs were done at 25.0 ± 0.1 °C in 1.00 mL of 0.1 M sodium phosphate buffer, pH 7.24 (open circles) or pD 7.73 (closed circles). Each reaction contained 0.1 M NaCl, 50 μg of heparin, 5.3 μg of LpL, 3.3% v/v CH_3CN, and the indicated concentrations (as reciprocals) of PNPB. Inset: Solvent isotope effects as a function of fractional saturation, $f_S = [S]/(K_m + [S])$, of LpL.

In eq 2, $V_{i,lim}$ is the limiting initial velocity at high pL. The linear transform of eq 2 is

$$V_i^{-1} = \frac{[H^+]}{V_{i,lim}K_a} + V_{i,lim}^{-1} \quad (3)$$

Weighted linear least-squares fits to eq 3, with weighting factors proportional to V_i^4, were used to calculate K_a and $V_{i,lim}$. The form of the weighting factors was determined according to the general procedures described by Cleland (1967).

Results

The solvent isotope effect probe of enzyme catalytic mechanism and transition-state structure has enjoyed considerable success in recent years [see Schowen (1978) for examples]. However, whenever solvent isotope effects are measured on an enzyme reaction for the first time, it must be established that effects on rate do not arise from a general medium effect of D_2O on the stability and/or gross conformation of the enzyme. That the solvent isotope effects reported in this paper on the rates of LpL-catalyzed hydrolysis of water-soluble *p*-nitrophenyl esters are effects on chemical steps of catalysis is established by three different types of experiments: (1) solvent deuterium isotope effects on K_m and V_{max}; (2) relative stability of LpL in H_2O buffers vs. D_2O buffers; (3) insensitivity of the solvent deuterium isotope effect to change in reaction temperature.

Figure 1 contains linear fits of data to the Lineweaver–Burk transform of the Michaelis–Menten equation for LpL-catalyzed hydrolysis of PNPB. The calculated values of V_{max} and K_m and the corresponding solvent isotope effects are collected in Table I, as are similar results for LpL-catalyzed hydrolysis of PNPA. The data of Table I demonstrate that V_{max} and V_{max}/K_m are sensitive to solvent isotopic substitution but that K_m is not greatly affected. Solvent isotope effects for hydrolysis of PNPB were measured by using two different LpL preparations. Despite the fact that the K_m's and V_{max}'s for the two preparations are different, the corresponding solvent isotope effects are equal within experimental error. The insensitivity of K_m to substitution of D_2O for H_2O supports the idea that rate reduction in D_2O vs. H_2O is an effect on reaction kinetics,

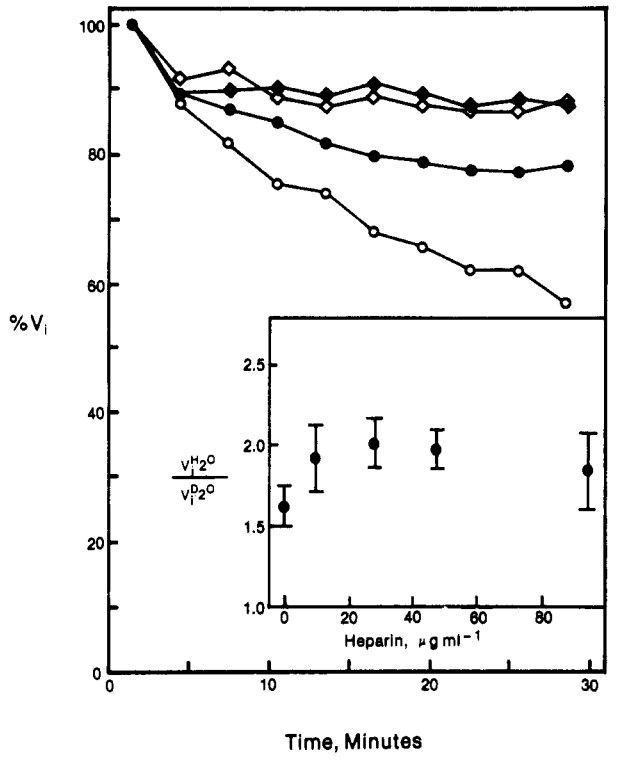


FIGURE 2: Stability curves for LpL at 25 °C and pH 7.24 (open symbols) or pD 7.73 (closed symbols). Each determination was performed in 1.00 mL of 0.1 M sodium phosphate buffer containing 0.1 M NaCl, 1% v/v CH_3CN, 3.4 μg of LpL, 0.1 mM PNPB, and 0 (circles) or 10 μg (diamonds) of heparin. Velocity remaining at various times after injection of LpL was determined from the instantaneous slope of the respective time course and expressed as $\%V_i$ (percent velocity remaining), using the velocity over the first 3 min of the 30-min time course as the 100% reference. Each velocity is the mean of at least two determinations. Inset: Solvent deuterium isotope effects on the initial velocity of LpL-catalyzed hydrolysis of PNPB at various heparin concentrations. Conditions were as above, except that each run contained 6.8 μg of LpL, 0.5 mM PNPB, and the indicated amounts of heparin. Each point is the ratio of the means of at least three determinations in H_2O and D_2O, respectively. Error limits were calculated as described in footnote *a* of Table I.

rather than an effect on enzyme stability or gross conformation.

LpL is notoriously unstable in aqueous buffers at 37 °C in the absence of a lipid interface (Bengtsson & Olivecrona, 1980). This instability is also noted but is much less pronounced in aqueous buffers at 25 °C, as Figure 2 shows for LpL-catalyzed hydrolysis of PNPB. The enzyme loses >40% of its activity in buffered H_2O in the absence of the stabilizing agent heparin (Iverius et al., 1972) but is *more stable* in buffered D_2O. When the reaction medium contains 10 μg/mL heparin, LpL retains 90% of activity after 30 min, and the stability plots in H_2O and D_2O virtually superimpose. A further increase in heparin concentration neither affords greater protection of LpL activity nor changes the relative stability profiles in H_2O and D_2O. In additional experiments reported in this paper, complications in interpretation of solvent isotope effects for LpL-catalyzed hydrolysis of water-soluble *p*-nitrophenyl esters are avoided by determining initial rates over the first 60 s of time courses, where the enzyme activity is near 100%, and by carrying out reactions with heparin added. The results plotted in the inset of Figure 2 show that increasing heparin concentration has no effect on the magnitude of the solvent isotope effect. The cumulative indication of these stability studies is that solvent deuterium isotope effects on LpL reactions are effects on reaction dynamics and not on enzyme stability. The inset of Figure 3 shows that,

Table I: Solvent Isotope Effects for LpL-Catalyzed Hydrolysis of *p*-Nitrophenyl Esters[a]

substrate	$10^7 V_{max}$ ($M\ s^{-1}$)	K_m (mM)	$V_{max}^{H_2O}/V_{max}^{D_2O}$	$(V_{max}/K_m)^{H_2O}/(V_{max}/K_m)^{D_2O}$
PNPA	7.0 ± 0.2[b]	0.76 ± 0.03[b]	1.83 ± 0.06	2.21 ± 0.08
	3.83 ± 0.08[c]	0.92 ± 0.03[c]		
PNPB	3.10 ± 0.05[d]	0.45 ± 0.01[d]	1.89 ± 0.05	1.72 ± 0.05
	1.64 ± 0.03[e]	0.41 ± 0.01[e]		
PNPB	10.4 ± 0.3[f]	0.78 ± 0.02[f]	1.82 ± 0.09	1.68 ± 0.04
	5.7 ± 0.2[g]	0.72 ± 0.03[g]		

[a] All measurements were made at 25.0 ± 0.1 °C. Error limits of V_{max} were calculated from the standard errors of intercepts of linear least-squares analyses of Lineweaver–Burk plots. K_m was calculated by dividing the slope by the intercept of linear least-squares analyses of Lineweaver–Burk plots. Error limits of K_m and of isotope effects (ratios) were calculated according to (error of ratio/ratio) = [(error of numerator/numerator)^2 + (error of denominator/denominator)^2]$^{1/2}$. Error limits of isotope effects for V_{max}/K_m were calculated by using this formula and the error limits of linear least-squares estimates of slopes of Lineweaver–Burk plots. [b] Reactions were done at pH 8.36 in 1.00 mL of 0.01 M Tris buffer that contained 0.1 M NaCl, 50 μg of heparin, 8 μg of LpL, and 2.8% (v/v) CH_3CN. [c] Same as footnote *b*, but in pD 8.94 Tris buffer. [d] Reactions were done at pH 7.24 in 1.00 mL of 0.1 M sodium phosphate buffer that contained 0.1 M NaCl, 50 μg of heparin, 5.3 μg of LpL, and 3.3% (v/v) CH_3CN. [e] Same as footnote *d*, but in pD 7.73 sodium phosphate buffer. [f] Same as footnote *d*, but in 1.00 mL of pH 7.30 sodium phosphate buffer that contained 10.6 μg of LpL and 1.9% (v/v) CH_3CN. These reactions used a different LpL preparation than those of footnotes *b–e*. [g] Same as footnote *f*, but in pD 7.83 sodium phosphate buffer.

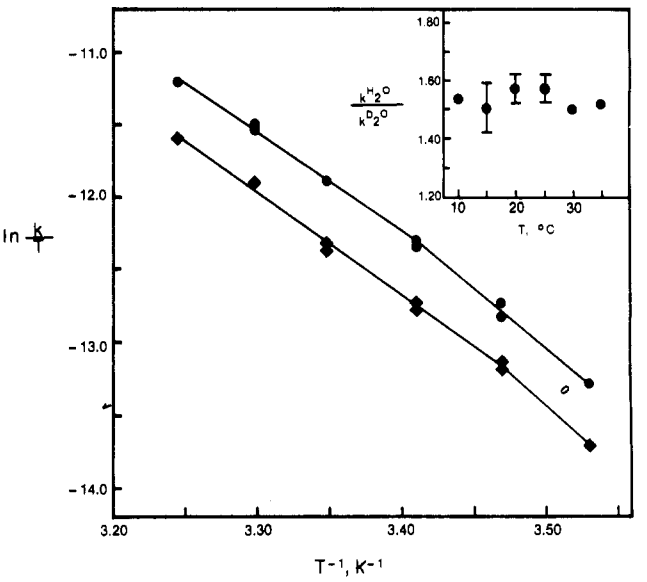


FIGURE 3: Eyring plots for LpL-catalyzed hydrolysis of PNPB in H_2O at pH 7.30 (circles) and in D_2O at pD 7.83 (diamonds). Duplicate determinations were done at each temperature in each solvent. Each reaction contained, in 1.00 mL of the respective buffer, 0.1 M NaCl, 100 μg of heparin, 2% (v/v) CH_3CN, 1.9×10^{-5} M PNPB ($=K_m/24$), and 31.8 μg of LpL. Under these conditions, reactions are first order, so that time course data were fit by nonlinear least-squares to the first-order function $A = (A_0 - A_\infty)e^{-kt} + A_\infty$, where t is time, k is the first-order rate constant V_{max}/K_m, and A_0 and A_∞ are the absorbances at time 0 and at infinite reaction time, respectively. Inset: Solvent isotope effects for LpL-catalyzed hydrolysis of PNPB as a function of temperature. Error limits were calculated as described in footnote *a* of Table I.

within experimental error, the solvent isotope effect is independent of temperature over the range 10–35 °C. Since LpL loses activity more rapidly as temperature is increased (Bengtsson & Olivecrona, 1980; Iverius et al., 1972), the temperature insensitivity of solvent isotope effects for LpL catalysis supports above-stated conclusions concerning the origins of the effects. Moreover, the Eyring plots (Glasstone

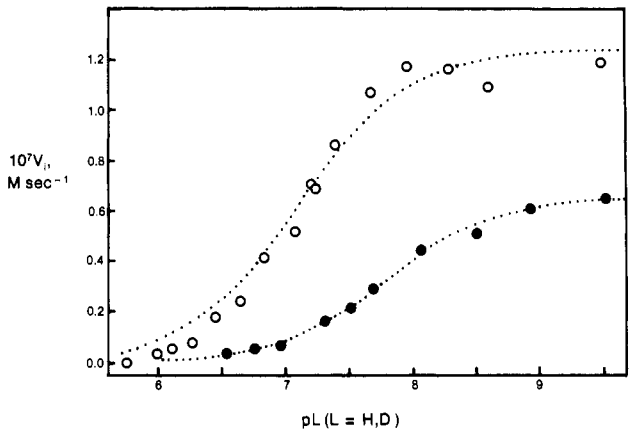


FIGURE 4: pL–rate (L = H or D) profiles for LpL-catalyzed hydrolysis of PNPB. Each run was performed at 25 °C and at the indicated pL in 1.00 mL of D_2O buffer (closed circles) or H_2O buffer (open circles) containing 0.1 M NaCl, 10 μg of heparin, 5.3 μg of LpL, 1% (v/v) CH_3CN, and 0.1 mM PNPB. Buffers were 0.1 M sodium phosphate (pL 5.5–8.1), 0.01 M Tris (pL 8.2–9), and 0.1 M Na_2HPO_4/Na_2CO_3 (pL >9). Each point is the mean of at least two determinations. Curvilinear lines are fits of the profiles to eq 2 of the text.

et al., 1941) of Figure 3 are biphasic, with downward breaks occurring near 20 °C in H_2O and near 15 °C in D_2O. The high-temperature segments of the Eyring plots yield activation parameters that are equal within experimental error: $\Delta H^{\ddagger}$ = 14.0 ± 0.2 and 13.5 ± 0.3 kcal mol^{-1} in D_2O and H_2O, respectively; $\Delta S^{\ddagger}$ = −1.5 ± 0.8 and −2 ± 1 eu in D_2O and H_2O, respectively. The similarity of the activation energetics in H_2O and D_2O indicates that the mechanism of LpL-catalyzed hydrolysis of PNPB in the two solvents is the same. Biphasic Eyring plots have been reported for LpL-catalyzed hydrolysis of PNPA and PNPB and ascribed to temperature-dependent changes in the rate-determining step (Quinn et al., 1982). The $\Delta H^{\ddagger}$ of 12.1 ± 0.3 kcal mol^{-1} for LpL-catalyzed hydrolysis of PNPB in H_2O calculated by Quinn et al. (1982) from the high-temperature segment of the Eyring plot compares well with the value reported herein.

Figure 4 shows the pH and pD–rate profiles for LpL-catalyzed hydrolysis of PNPB. Both profiles increase sigmoidally with increasing pL (L = H or D) and are well described mathematically by eq 2 of Data Reduction Procedures under Materials and Methods. Weighted linear fits to eq 3, from which the curves of Figure 4 were calculated, yielded the following results: $pK_a^{H_2O}$ = 7.10 ± 0.01, $pK_a^{D_2O}$ = 7.795 ± 0.007, $V_{lim}^{H_2O}$ = (1.26 ± 0.02) × 10^{-7} M s^{-1}, $V_{lim}^{D_2O}$ = (6.59 ± 0.07) × 10^{-8} m s^{-1}. The corresponding solvent isotope effect is $V_{lim}^{H_2O}/V_{lim}^{D_2O}$ = 1.97 ± 0.03 (corrected for isotopic dilution of D_2O buffers). The pK_a calculated from the pH–rate profile is numerically similar to the pK_a determined by Fielding (1973) of 6.8–7.0 for rat post-heparin plasma and rat adipose tissue LpL-catalyzed hydrolysis of emulsified trioleylglycerol. Both profiles in Figure 4 plateau at alkaline pLs, which contrasts with the pH–rate profile described by Quinn et al. (1983), which had an apparent maximum at pH 8.3. This is likely due to the fact that 0.1 M Tris, used to buffer reactions at pH >8 in the previous study, produces 20–30% inhibition of LpL-catalyzed hydrolysis of PNPB.[2] This complication was avoided in the work reported in this paper by using 0.01 M Tris buffers.

Vainio et al. (1982) found benzeneboronic acid to be a potent inhibitor of LpL-catalyzed hydrolysis of trioleylglycerol, with a K_i of 8.9 μM at pH 7.4 and 37 °C. Dixon plots (Segel, 1975) show that benzeneboronic acid is also a competitive inhibitor of LpL-catalyzed hydrolysis of PNPB. The K_i calculated from the intersection of the Dixon plots is 6.9 ± 0.4 μM.

Discussion

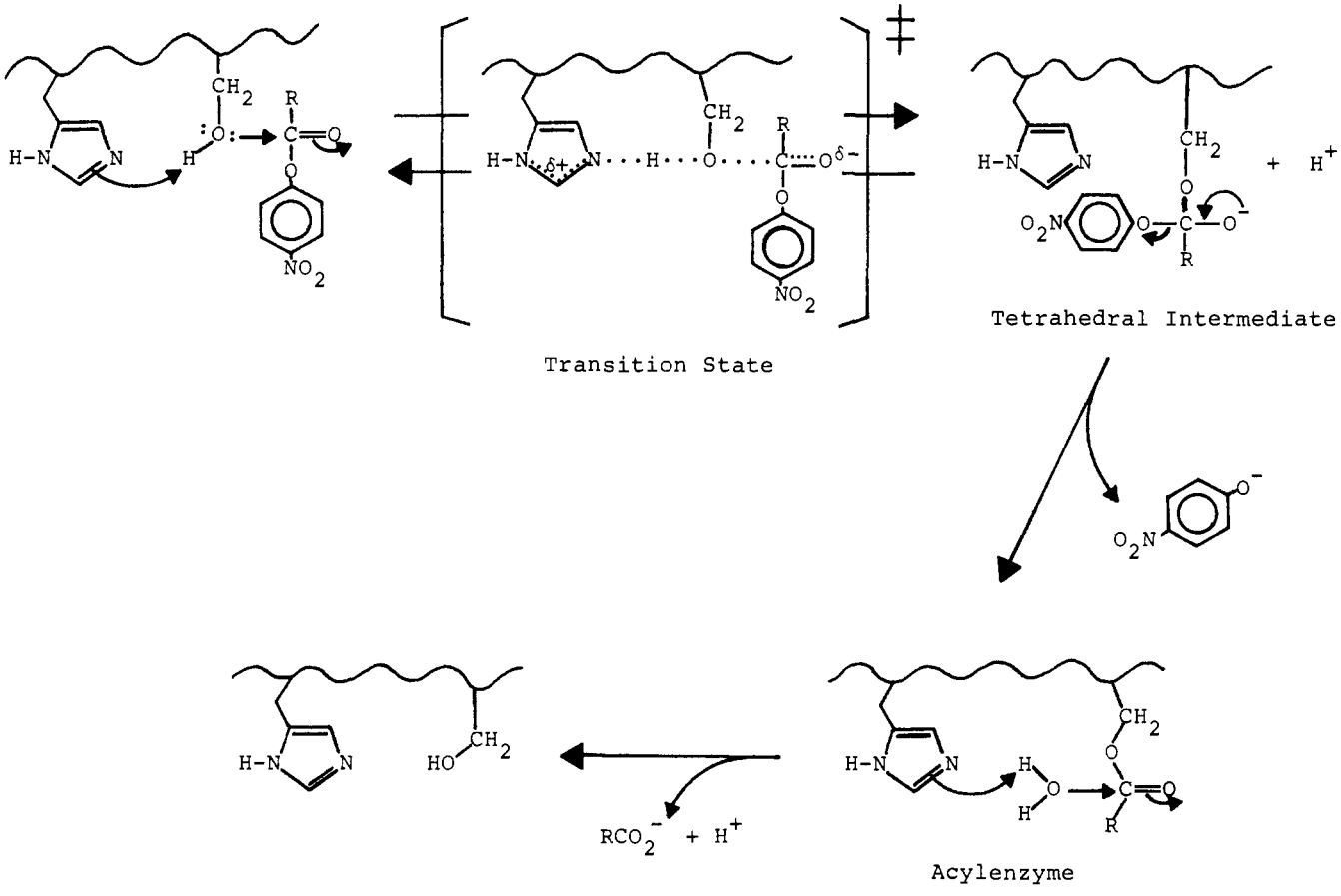
In a recent review, Quinn et al. (1983) suggested that the weight of experimental evidence supports an acylenzyme mechanism for LpL catalysis that is similar to the mechanisms of serine proteases (Stroud, 1974). This view is supported by the observation that [^{3}H]PMSF, an irreversible inhibitor of LpL-catalyzed hydrolysis of PNPB and trioleylglycerol (Quinn et al., 1982, 1983), binds to LpL to form a 1:1 complex. The tritiated peptide resulting from trypsinization of the inhibited enzyme had the composition 1 Asp/2 Ser/1 Glu/2 Gly/1 His/1 Lys[3] and hence contains the amino acids of the Gly-Asp-Ser-Gly sequence of the active sites of various serine proteases (Lecroisey & Keil, 1983). Vainio et al. (1982) have shown that benzeneboronic acid is a potent competitive inhibitor of LpL catalysis, with a K_i of 8.9 μM at pH 7.4 and 37 °C. Boronic acids are known to inhibit serine proteases by suffering nucleophilic addition to the boron atom of the active-site serine to form an adduct that resembles the high-energy tetrahedral intermediates of the catalytic cycle (Blow, 1976; Rawn & Lienhard, 1974). Benzeneboronic acid is also a potent competitive inhibitor of LpL-catalyzed hydrolysis of PNPB, which supports the assignment of Quinn et al. (1982) of a single LpL active site for the hydrolysis of triacylglycerols and of water-soluble *p*-nitrophenyl esters. Vainio et al. (1982) interpret their results as indicating the presence of serine and histidine at LpL's active site and as support for an acylenzyme mechanism for LpL action. Vainio et al. (1983a,b) suggest that deacylation of a fatty acyl–LpL intermediate is the rate-determining step for hydrolysis of triacylglycerols. They propose a mechanism for apoC-II activation of the enzyme that involves nucleophilic attack of Ser-60 of apoC-II on the rate-limiting acyl-LpL intermediate. Fielding (1973) suggested the presence of an active-site histidine to explain the pK_a = 6.8–7.0 on the acid limbs of the pH–rate profiles for LpL-catalyzed hydrolysis of emulsified trioleylglycerol. The enthalpy of ionization of the titrating active-site group was 5.5 kcal mol^{-1}, which Fielding suggests supports the assignment of an active-site histidine. Sugiura & Oikawa (1980) demonstrated that loss of activity of LpL from *Pseudomonas fluorescens* on photooxidation in the presence of methylene blue is accompanied by the loss of a histidine residue. The rate of photooxidation increased with pH and yielded a pK_a = 7. These results also support a catalytically important histidine at LpL's active site.

The results in this paper add precedent to the assignment of a serine esterase mechanism for LpL catalysis. Mathematical analysis of the pH–rate profile of Figure 4 yields a pK_a = 7.10 ± 0.01. This pK_a is similar to those reported by Fielding (1973) and Sugiura & Oikawa (1980) for various LpL reactions. Moreover, the pK_a of the LpL-catalyzed hydrolysis of PNPB shifts in an alkaline direction by ΔpK_a = 0.70 ± 0.02 upon transfer of the reaction to D_2O, as Figure 4 shows. This solvent deuterium isotope effect on pK_a is that expected for ionization of weak organic acids, which have ΔpK_a's of 0.5 ± 0.2 (Jencks, 1969). The good fits of the pL–rate profiles of Figure 4 to eq 2 indicate that reaction velocity depends on the basic form of a single active-site amino acid side chain. The consistent observation of solvent isotope effects of 1.5–2.2 with varying temperature, pH, enzyme

[2] D. M. Quinn, unpublished results.

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

Scheme I: Proposed Mechanism for LpL Catalysis



stability, substrate structure (PNPA vs. PNPB), and substrate concentration is firm evidence that proton transfer is a structural element of transition states for LpL-catalyzed hydrolysis of water-soluble esters. The magnitude of the solvent isotope effect is reminiscent of those of 1.5–3 for a large number of serine protease catalyzed (Schowen, 1978) and acetylcholinesterase-catalyzed reactions (Rosenberry, 1975a,b).

The results reported and the literature precedent cited herein allow the postulation of a chemical model for LpL catalysis, which is shown in Scheme I for *p*-nitrophenyl ester substrates. This scheme is similar in many respects to the mechanism for chymotrypsin action that Blow (1976) has described. The LpL catalytic mechanism involves two major reactions processes: the acylation process culminates in the acylenzyme intermediate; the acylenzyme hydrolyzes with proton transfer assistance from the active-site histidine. V_{max}/K_m is the rate constant for the rate-determining step through the first irreversible event of the catalytic mechanism (Hegazi et al., 1978) and hence monitors LpL acylation. Furthermore, the rate-determining transition state for acylation is probably that for formation of the tetrahedral intermediate. The breakdown of the tetrahedral intermediate involves expulsion of *p*-nitrophenoxide, which should not require proton transfer catalysis due to the low $pK_a = 6.98$ of *p*-nitrophenol. The transition state for the formation of the tetrahedral intermediate is stabilized by a general base proton bridge between the nucleophilic serine and the active-site histidine. This suggestion is consistent with the pK_a of 7.10 determined from the pH–rate profile of Figure 4, and with literature precedents discussed earlier. Because the proposed acylation transition state involves a general base proton bridge as a dynamical element, the acylation stage of LpL catalysis is accompanied by a solvent isotope effect of 1.5–2.2.

The pL–rate profiles of Figure 4 were determined by measuring initial rates at [PNPB] $< 0.2K_m$. Therefore, the initial rates depend primarily on V_{max}/K_m, so that pK_a's calculated from the profiles are those for free LpL (Quinn et al., 1980). The $pK_a = 7.10$ is thus a measure of the acid ionization behavior of the LpL active site. Furthermore, LpL-catalyzed hydrolysis of PNPB is a homogeneous reaction (i.e., the substrate is monomolecularly dispersed), so that interpretation of pK_a's is not fraught with the complications associated with the pH–rate behavior of interfacial LpL catalysis.

Because LpL-catalyzed hydrolysis of lipid substrates and water-soluble *p*-nitrophenyl esters occurs at the same active site, the pH–rate effects and solvent isotope effects reported in this paper are of significance for the LpL mechanism in general. Similarity of the chemical mechanisms of LpL-catalyzed hydrolysis of water-soluble substrates and of interfacial LpL catalysis can be further established by measuring solvent isotope effects for interfacial LpL catalysis. Such studies are under way in the author's laboratory.

Registry No. LpL, 9004-02-8; PNPA, 830-03-5; PNPB, 2635-84-9; benzeneboronic acid, 98-80-6; deuterium, 7782-39-0.

References

Bengtsson, G., & Olivecrona, T. (1980) *Eur. J. Biochem. 106*, 549–555.

Blow, D. M. (1976) *Acc. Chem. Res. 9*, 145–152.

Breckenridge, W. C., Little, J. A., Steiner, G., Chow, A., & Poapst, M. (1978) *N. Engl. J. Med. 298*, 1265–1273.

Cleland, W. W. (1967) *Adv. Enzymol. Relat. Areas Mol. Biol. 29*, 1–32.

Cox, D. W., Breckenridge, W. C., & Little, J. A. (1978) *N. Engl. J. Med. 299*, 1421–1424.

Fielding, C. J. (1973) *Biochim. Biophys. Acta 316*, 66–75.

Glasstone, S., Laidler, K. J., & Eyring, H. (1941) *The Theory of Rate Processes*, McGraw-Hill, New York.
Havel, R. J., & Gordon, R. S. (1960) *J. Clin. Invest. 39*, 1777–1790.
Hegazi, M. F., Quinn, D. M., & Schowen, R. L. (1978) in *Transition States of Biochemical Processes* (Gandour, R. D., & Schowen, R. L., Eds.) pp 355–428, Plenum Press, New York.
Iverius, P. H., Lindahl, U., Egelrud, T., & Olivecrona, T. (1972) *J. Biol. Chem. 247*, 6610–6616.
Jencks, W. P. (1969) *Catalysis in Chemistry and Enzymology*, pp 250–253, McGraw-Hill, New York.
Kannel, W. B., Castelli, W. P., & Gordon, T. (1979) *Ann. Intern. Med. 90*, 85–91.
Kinnunen, P. K. J. (1977) *Med. Biol. 55*, 187–191.
Lecroisey, A., & Kiel, B. (1983) *Biochem. Biophys. Res. Commun. 112*, 907–910.
Matsuoka, N., Shirai, K., & Jackson, R. L. (1980) *Biochim. Biophys. Acta 620*, 308–316.
Miller, G. J. (1980) *Annu. Rev. Med. 31*, 97–108.
Quinn, D. M., Elrod, J. P., Ardis, R., Friesen, P., & Schowen, R. L. (1980) *J. Am. Chem. Soc. 102*, 5358–5365.
Quinn, D. M., Shirai, K., Jackson, R. L., & Harmony, J. A. K. (1982) *Biochemistry 21*, 6872–6879.
Quinn, D. M., Shirai, K., & Jackson, R. L. (1983) *Prog. Lipid Res. 22*, 35–78.
Rawn, J. D., & Lienhard, G. E. (1974) *Biochemistry 13*, 3124–3130.
Rosenberry, T. L. (1975a) *Adv. Enzymol. Relat. Areas Mol. Biol. 43*, 103–218.
Rosenberry, T. L. (1975b) *Proc. Natl. Acad. Sci. U.S.A. 72*, 3834–3838.
Salomaa, P., Schaleger, L. L., & Long, F. A. (1964) *J. Am. Chem. Soc. 86*, 1–7.
Schowen, K. B. J. (1978) in *Transition States of Biochemical Processes* (Gandour, R. D., & Schowen, R. L., Eds.) pp 225–283, Plenum Press, New York.
Segel, I. H. (1975) *Enzyme Kinetics*, pp 109–111, Wiley, New York.
Shirai, K., & Jackson, R. L. (1982) *J. Biol. Chem. 257*, 1253–1258.
Stroud, R. M. (1974) *Sci. Am. 231*, 74–88.
Sugiura, M., & Oikawa, T. (1980) *Chem. Pharm. Bull. 28*, 2803–2806.
Tall, A. R., & Small, D. M. (1980) *Adv. Lipid Res. 17*, 1–51.
Vainio, P., Virtanen, J. A., & Kinnunen, P. K. J. (1982) *Biochim. Biophys. Acta 711*, 386–390.
Vainio, P., Virtanen, J. A., Kinnunen, P. K. J., Gotto, A. M., Sparrow, J. T., Pattus, F., Bougis, P., & Verger, R. (1983a) *J. Biol. Chem. 258*, 5477–5482.
Vainio, P., Virtanen, J. A., Sparrow, J. T., Gotto, A. M., & Kinnunen, P. K. J. (1983b) *Chem. Phys. Lipids 33*, 21–32.
Zilversmit, D. B. (1977) *Adv. Exp. Med. Biol. 109*, 45–59.
Zilversmit, D. B. (1979) *Circulation 60*, 473–485.

Clostridium histolyticum Collagenase: Development of New Thio Ester, Fluorogenic, and Depsipeptide Substrates and New Inhibitors†

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ABSTRACT: A new series of thio ester, depsipeptide, and peptide substrates have been synthesized for the bacterial enzyme *Clostridium histolyticum* collagenase. The hydrolysis of the depsipeptide substrate was followed on a pH stat, and thio ester hydrolysis was measured by inclusion of the chromogenic thiol reagent 4,4′-dithiopyridine in the assay mixture. The best thio ester substrate, Boc-Abz-Gly-Pro-Leu-SCH_2CO-Pro-Nba, had a k_{cat}/K_M of 63 000 M^{-1} s^{-1}, while several shorter thio ester sequences were inactive as substrates. In general, the peptide analogues of all the reactive thio ester substrates were shown to be hydrolyzed 5–10 times faster by collagenase. In one case (Z-Gly-Pro-Leu-Gly-Pro-NH_2) where a comparison was made, the peptide substrate was respectively 8- and 106-fold more readily hydrolyzed than the corresponding thio ester and ester substrates. Cleavages of the two fluorescence-quench substrates Abz-Gly-Pro-Leu-Gly-Pro-Nba and Abz-Gly-Pro-Leu-SCH_2CO-Pro-Nba could be easily followed fluorogenically since a 5–10-fold increase in fluorescence occurred upon hydrolysis. The fluorescent peptide substrate is the best synthetic substrate known for *C. histolyticum* collagenase with a k_{cat}/K_M value of 490 000 M^{-1} s^{-1}. A series of new reversible inhibitors were developed by the attachment of zinc ligating groups (hydroxamic acid, carboxymethyl, and thiol) to various peptide sequences specific for *C. histolyticum* collagenase. The shorter peptides designed to bind to either the P_3–P_1 or P_1'–P_3' subsites were poor to moderate inhibitors. The thiol $HSCH_2CH_2CO$-Pro-Nba had the lowest K_I (0.02 mM). Elongation of *N*-hydroxy peptide sequences to interact with the P_3–P_3' subsites of the enzyme failed to yield better inhibitors. None of the potential irreversible inhibitor structures, which contained $ClCH_2CO$— or CH_2=CH—CO— groups attached to peptides, proved to be effective.

Collagenases are a small group of highly specific proteases capable of causing hydrolytic cleavage in the triple-helical region of the collagen molecule. In contrast to tissue collagenases, which cleave the collagen helix at a single site, bacterial collagenase will make multiple cleavages. *Clostridium histolyticum* collagenase (EC 3.4.24.3) is one of the most widely studied bacterial enzymes capable of cleaving collagen.

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