Interfacial Reaction Dynamics and Acyl-enzyme Mechanism for Lipoprotein Lipase-catalyzed Hydrolysis of Lipid p-Nitrophenyl Esters*

Rebecca A. Burdette and Daniel M. Quinn†

From the Department of Chemistry, The University of Iowa, Iowa City, Iowa 52242

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The fatty acyl (lipid) p-nitrophenyl esters p-nitrophenyl caprylate, p-nitrophenyl laurate and p-nitrophenyl palmitate that are incorporated at a few mol % into mixed micelles with Triton X-100 are substrates for bovine milk lipoprotein lipase. When the concentration of components of the mixed micelles is approximately equal to or greater than the critical micelle concentration, time courses for lipoprotein lipase-catalyzed hydrolysis of the esters are described by the integrated form of the Michaelis-Menten equation. Least square fitting to the integrated equation therefore allows calculation of the interfacial kinetic parameters $K^*$ and $V_{max}$ from single runs. The computational methodology used to determine the interfacial kinetic parameters is described in this paper and is used to determine the intrinsic substrate fatty acyl specificity of lipoprotein lipase catalysis, which is reflected in the magnitude of $K^*_{ac}$/$K^*_{w}$ and $K^*_{ac}$. The results for interfacial lipoprotein lipase catalysis, along with previously determined kinetic parameters for the water-soluble esters p-nitrophenyl acetate and p-nitrophenyl butyrate, indicate that lipoprotein lipase has highest specificity for the substrates that have fatty acyl chains of intermediate length (i.e. p-nitrophenyl butyrate and p-nitrophenyl caprylate). The fatty acid products do not cause product inhibition during lipoprotein lipase-catalyzed hydrolysis of lipid p-nitrophenyl esters that are contained in Triton X-100 micelles. The effects of the nucleophiles hydroxylamine, hydrazine, and ethylenediamine on $K^*$ and $V_{max}$ for lipoprotein lipase-catalyzed hydrolysis of p-nitrophenyl laurate are consistent with trapping of a lauryl-lipoprotein lipase intermediate. This mechanism is confirmed by analysis of the product lauryl hydroxamate when hydroxylamine is the nucleophile. Hence, lipoprotein lipase-catalyzed hydrolysis of lipid p-nitrophenyl esters that are contained in Triton X-100 micelles occurs via an interfacial acyl-lipoprotein lipase mechanism that is rate-limited by hydrolysis of the acyl-enzyme intermediate.

Lipoprotein lipase-catalyzed hydrolysis of lipid substrates is a heterogeneous biocatalytic process. The velocity of lipoprotein lipase reactions, like that of numerous reactions catalyzed by soluble enzymes at lipid-water interfaces, increases in a manner functionally described by the Michaelis-Menten equation as the analytical concentration of lipid substrate increases (1, 2). However, the kinetic parameters $K^*$ and $V_{max}$ do not bear the same relationship to the various microscopic steps of the catalytic mechanism as they do for single-substrate enzyme reactions of soluble enzymes and monomolecularly dispersed (i.e. water-soluble) substrates. This difference is illustrated by considering the Verger-deHaas model for interfacial enzyme catalysis (2).

$$E + M \xrightarrow{k_0} E^* + \frac{k_x}{k_j} ES^* \xrightarrow{k_{max}} E^* + P$$

**SCHEME I**

The definitions of terms in Scheme I are $E = $ free lipoprotein lipase; $E^* = $ lipoprotein lipase bound to the substrate-containing particle interface, but with unoccupied active sites (the penetration complex); $ES^* = $ Michaelis complex of lipoprotein lipase and substrate formed at the interface; $K^* = $ concentration of substrate in the interface in mole fraction; $M = $ the monomolecularly dispersed or molecularly dispersed, water-soluble) substrate. This difference in $K^*$ and $V_{max}$ for interfacial lipoprotein lipase catalysis, but $K^*$ and $V_{max}$ for interfacial lipoprotein lipase catalysis, but

$$V_i = \frac{V_{max}}{K_{max}} \frac{(x_d[M])}{(x_d[M])}$$

In Equation 1, [M] is the analytical concentration of all the components that comprise the substrate-containing particle (N.B., Verger and deHaas use interface area per volume). Equation 1 shows that velocity increases as [M] increases, because the total interface area of substrate-containing particles concomitantly increases and eventually the enzyme is saturated by interface (i.e. all enzyme is in the $E^*$ form). However, $x_d$, which measures substrate concentration in the particles, does not change. The kinetic parameters of Equation 1 are similarly complicated.

$$V_{max} = \frac{V_{max} \times x_d}{K^*_a + x_d} \quad K^*_a = \frac{k_d}{k_j} \quad K^*_a = \frac{k_d}{k_j}$$

In Equations 2, $K^*_a = (k_d + k_{cat})/k_j$ and $V_{max} = k_{cat}[E^*]_i$, where $[E^*]_i = [E^*] + [ES^*]$. $V_{max}$ depends not only on $V_{max}$, the intrinsic $V_{max}$ for interfacial lipoprotein lipase catalysis, but also on the fractional saturation, $x_d/(K^*_a + x_d)$, of active sites of lipoprotein lipase that is interface bound (1, 3). $K^*_a$ depends on the dissociation constant of the penetration complex, $k_d/k_j$, on the fractional saturation of lipoprotein lipase active sites, and on $K^*_a$. Hence, the values of the interfacial Michaelis-Menten parameters $V_{max}$ and $K^*_a$ cannot be had...
Interfacial Lipoprotein Lipase Reaction Dynamics

Clear methods for directly determining the interfacial Michaelis-Menten parameters \( k_{+2} \) and \( V_{\text{max}} \) would be of great value in determining how such factors as pH, temperature, substrate structure, added nucleophiles, and the physiological activator apolipoprotein C-II affect the molecular dynamics of lipoprotein lipase catalysis. This paper describes the development of micellar substrates for lipoprotein lipase that consist of Triton X-100 and the \( p \)-nitrophenyl esters of caprylic, lauric, or palmitic acid. Because lipoprotein lipase-catalyzed hydrolysis of these esters releases \( p \)-nitrophenoxide, which absorbs light at 400 nm, reaction of the micellar substrates can be followed continuously by spectrophotometry. Time courses for lipoprotein lipase-catalyzed hydrolysis of the lipid \( p \)-nitrophenyl esters are first-order at concentrations below the critical micelle concentration of the mixed micelles. However, at concentrations near and above the critical micelle concentration, the kinetics become mixed-order and are well described by the integrated form of the Michaelis-Menten equation. Hence, least-squares analysis of the mixed-order time courses allows calculation of \( V_{\text{max}} \) and \( k_{+2} \). This method is used herein to characterize the mechanism of interfacial lipoprotein lipase-catalyzed hydrolysis of lipid \( p \)-nitrophenyl esters.

MATERIALS AND METHODS AND RESULTS

**DISCUSSION**

Physiological lipoprotein lipase catalysis involves the hydrolysis of triacylglycerols and phospholipids of the triacylglycerol-rich lipoproteins, very low density lipoproteins and chylomicrons, and lipoprotein lipase are bound to Triton X-100 micelles. Hence, like lipoprotein lipase turnover of triacylglycerols of lipoproteins, the turnover of lipid \( p \)-nitrophenyl esters is an interfacial lipolytic reaction. Above the critical micelle concentration of the mixed micelles, the kinetics of lipoprotein lipase-catalyzed hydrolysis of lipid \( p \)-nitrophenyl esters are described by the integrated Michaelis-Menten equation. This fact opens interfacial lipoprotein lipase catalysis difficult to characterize for the reasons discussed in the Introduction. In this manuscript we describe the development and characterization of a novel lipoprotein lipase reaction. The hydrolysis of lipid \( p \)-nitrophenyl esters that are contained in mixed micelles with Triton X-100. This system offers advantages for the study of interfacial lipoprotein lipase catalysis. (a) The reaction is biomimetic. Both lipid \( p \)-nitrophenyl ester and lipoprotein lipase are bound to Triton X-100 micelles (cf. Fig. 1). Hence, like lipoprotein lipase turnover of triacylglycerols of lipoproteins, the turnover of lipid \( p \)-nitrophenyl esters is an interfacial lipolytic reaction. (b) Above the critical micelle concentration of the mixed micelles, the kinetics of lipoprotein lipase-catalyzed hydrolysis of lipid \( p \)-nitrophenyl esters are described by the integrated Michaelis-Menten equation. This fact opens interfacial lipoprotein lipase catalysis to the kind of scrutiny that has long been applied to homogeneous enzyme catalysis. Hendrickson and Dennis (21, 22) have developed an interfacial kinetics model for phospholipase \( A_2 \)-catalyzed hydrolysis of dithioester analogs of phosphatidylcholine and phosphatidylethanolamine that are contained in Triton X-100 micelles. Hence, our use of Triton X-100 mixed micelles as model substrates for lipoprotein lipase is not without precedent in the literature of lipolytic enzyme mechanisms.

A hallmark of lipoprotein lipase-catalyzed hydrolysis of triacylglycerols that are contained in emulsions or lipoproteins is product inhibition by fatty acids (13-17) in the absence of a fatty acid acceptor such as bovine serum albumin. The results reported herein are unusual in that fatty acid products do not inhibit the lipoprotein lipase reaction. Plots such as the inset of Fig. 2B show that lipoprotein lipase-catalyzed hydrolysis of lipid \( p \)-nitrophenyl esters is well described by the integrated Michaelis-Menten equation. Progressive product inhibition would be accompanied by systematic upward curvature of the plot. Lack of product inhibition is not peculiar to the composition of the micelle, since the interfacial kinetic parameters do not depend on the mole fraction of substrate in the micelle. The most surprising finding, however, is that addition of lauric acid to \( p \)-nitrophenyl laurate micelles activates the reaction. Hence, product inhibition in lipoprotein or emulsion substrates likely does not occur by mechanisms that involve competitive binding at the lipoprotein lipase active site or binding on an effector site that is distal from the active site (See Ref. 1 for a presentation of possible fatty acid inhibition mechanisms). Bengtsson and Olivecrona (14) showed that, in the absence of albumin, lipoprotein lipase synthesizes acylglycerols from \( [\text{H}] \)oleic acid in the presence of a trioleylglycerol/gum arabic emulsion. This is probably not the product inhibition mechanism for lipid \( p \)-nitrophenyl ester substrates because the \( p \)-nitrophenoxide product is neither lipophilic nor nucleophilic and does not accumulate in the micelle or participate in the reverse reaction. It is also possible that the reverse reaction does not occur because the fatty acid products rapidly diffuse away from the micelles. The linearity of the plot in the Fig. 2B inset is consistent with the lack of a reverse reaction. These considerations suggest that mixed micelles of Triton X-100 and lipid \( p \)-nitrophenyl esters are good systems for further probing the mechanism of product inhibition of lipoprotein lipase.

As mentioned earlier, lipoprotein lipase-catalyzed hydrolysis of mixed micellar lipid \( p \)-nitrophenyl esters is a biomimetic reaction. If this is so, the reaction ought to share some of the features of lipoprotein lipase-catalyzed hydrolysis of physiological substrates. Shinomiya et al. (23) studied the lipoprotein lipase-catalyzed hydrolysis of an homologous series of phosphatidylcholines in mixed micelles with Triton X-100. They found that in the absence of the activator apolipoprotein C-II the reaction velocity decreased with increasing fatty acyl chain length. Bengtsson and Olivecrona (24) found that bovine milk lipoprotein lipase more readily hydrolyzes trioc-tanoin than triolein when the lipids are contained in Triton X-100-stabilized emulsions. We have observed decreasing lipoprotein lipase activity with increasing chain length for the lipid \( p \)-nitrophenyl esters \( p \)-nitrophenyl caprylate, \( p \)-nitrophenyl laurate, and \( p \)-nitrophenyl palmitate, as reflected in both \( k_{+2} \) and \( V_{\text{max}}/k_{+2} \) values (cf. Table I). For the water-soluble substrates \( p \)-nitrophenyl acetate and \( p \)-nitrophenyl butyrate the data of Table I show that lipoprotein lipase prefers the longer acyl chain. These trends are consistent with highest intrinsic lipoprotein lipase specificity for fatty acyl chains of intermediate length. The fact that the trend in fatty acyl specificity for lipoprotein lipase-catalyzed hydrolysis of lipid \( p \)-nitrophenyl esters parallels that for hydrolysis of triacylglycerols and phosphatidylcholines suggests that the reaction
system described in this paper is indeed biomimetic. The plots of Fig. 4 show that the nucleophile hydroxylamine increases $K_m^*$ and $V_{\text{max}}$ by equal amounts, and hence the Lineweaver-Burk plots are parallel. Scheme II depicts a mechanism that is consistent with these kinetics. Nucleophilic attack by the active site serine (1, 19, 25) and consequent loss of $p$-nitrophenoxide produces the enzy-me intermediate. Hydroxylamine activates the reaction by providing an alternate route for decomposition of the acyl-lipoprotein lipase intermediate. This mechanism is an interfacial catalysis analog of the mechanism of nucleophilic activation of serine proteases (26, 27). For such a mechanism, a steady-state derivation of the kinetic parameters gives:

$$V_{\text{max}} = k_2^{*}[E]^*_{\text{T}} = (k_i + k_o [\text{Nu}]) [E]^*_{\text{T}}$$  

(8)

$$K_m^* = k_2^* k_i + k_o [\text{Nu}] k_o / k_i$$  

(9)

In these equations $\text{Nu}$ is hydroxylamine. $K_m^*$ in Equation 9 is the Michaelis constant for the interfacial lipoprotein lipase-substrate complex. $K_e^*$ contains $k_i$ and $k_o$ (not shown in Scheme II), which are, respectively, the rate constants for association and dissociation of substrate monomers at the lipoprotein lipase active site. Equations 8 and 9 are predicated on the concept that decomposition of the acyl-lipoprotein lipase intermediate is the rate-determining step. These equations predict linear and matching increases in $V_{\text{max}}$ and $K_m^*$ as $[\text{Nu}]$ increases. The plots of Fig. 4B show that this is the case. Chemical analysis of reaction products for hydroxylamine-activated lipoprotein lipase-catalyzed hydrolysis of $p$-nitrophenyl laurate (cf. "Results") also supports the mechanism of Scheme II. This is the first example of a kinetic signature of an acyl-enzyme mechanism for any lipoprotein lipase-catalyzed reaction. Therefore, micellar lipid $p$-nitrophenyl esters offer a system for which the molecular details of interfacial lipoprotein lipase catalysis are reasonably well defined.

The ability to measure the interfacial kinetic parameters $K_m^*$ and $V_{\text{max}}$ opens additional aspects of lipoprotein lipase reaction dynamics to scrutiny. For example, solvent isotope effects can determine whether interfacial lipoprotein lipase catalysis involves transition states that are stabilized by proton transfer (19). The effect of mechanism-based inhibitors on interfacial lipoprotein lipase reaction dynamics can be defined. Lipid $p$-nitrophenyl esters can be incorporated into different micelles, such as phosphatidylcholine micelles, which can provide a yet more biomimetic substrate system. These and other investigations of interfacial lipoprotein lipase catalysis are being pursued in our laboratory.

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SUPPLEMENTARY MATERIAL TO INTERFACIAL REACTION DYNAMICS AND ACYLTRANSFER MECHANISM FOR LIPOPROTEIN LIPOASE-CATALYZED HYDROLYSIS OF LIQUID P-NITROPHENYL CESTER.

REBECCA A. BURDOTTIE AND DANIEL M. QUINN

MATERIALS AND METHODS

Materials - LpL was purified from skimmed bovine milk by using previously described procedures (4,5). The purified enzyme showed a single band when assayed for homogeneity by SDS-PAGE, PNPC, PNPL, PNPP, blue dextran, the sodium salt of heparin from piscine intestinal mucosa, TX100, Sepharose CL-6B and rhodamine were purchased from Sigma Chemical Company and were used as received. All other materials were commercially available reagent-grade products.

Micellar Substrate Preparation and Characterization - Mixed micellar substrates were prepared by weighing on an analytical balance the requisite amounts of liquid-p-nitrophenyl-water and TX100. The mixture was stirred with a magnetic stirrer and a small cylindrical stirring bar until the mixture was completely dissolved in the TX100. Magnetic stirring was maintained and 0.1 M sodium phosphate buffer that contained NaCl (pHs are indicated in Figure legends) was slowly added to the mixture to a total volume of 30 mL. Outstanding of the substrate mixture was observed by way of optical detection. Fresh substrate solutions were prepared on the day of the experiment. The concentration of liquid p-nitrophenyl ester contained in mixed micellar solutions was determined by TLC analysis. The reaction mixture was extracted into the methanolic phase at pH 13 by an absorbivity constant of 21388. The rhodamine 6G-staining assay described by Carey & Small (6) was used to determine the CMC of micelles of TX100 and liquid p-nitrophenyl esters. TX100 micelles, with and without PNPL or LpL, were also characterized by gel filtration on a column of Sepharose CL-6B. Before sample application, the column was washed with at least two column volumes of 0.5 M TRIS buffer (pH 8.0) containing 0.5 M sodium phosphate buffer, pH 6.0, that contained 0.3 M NaCl, followed by equilibration with at least two column volumes of buffer. The rates of buffer exchange and equilibration were measured by the elution of blue dextran and p-nitrophenol, respectively. Micellar solutions followed by measuring absorbance at 526 nm. PNPL elution was determined by first adding 1 mL of each column fraction to 3.25 mL of water-water. The blank solution showed that p-nitrophenol that was produced by hydrolysis of PNPL in the absence of LpL was quantitatively extracted into the methanolic aqueous phase, while the lower CMC-7,5 phase contained the enzyme. PNPL was then determined by measuring the absorbance at 277 nm. The concentration of PNPL-catalyzed hydrolysis of lipid p-nitrophenyl esters was measured at 595 nm. In all cases, samples were greater than 20 mL. Substrate solutions prepared in these buffers were used for enzyme kinetics and micelle characterization.

Enzyme Kinetics and Data Reduction - Timecourses for absorbance increase at 400 nm for liquid-p-nitrophenyl ester were observed by using a Hewlett-Packard 8452A UV-visible spectrophotometer that was interfaced to an IBM Personal Computer. Data for reactions conducted below the CMC of the respective mixed micellar substrate were fit by nonlinear-least squares to the first-order reaction function of eq 3. A, B, and C are absorbance at time t, at t = 0 and at t, respectively. k' is the first-order rate constant and V is the initial substrate concentration.

The reaction substrates used were prepared as described above, the CMC, timecourses were described by the integrated form of the Michaelis-Menten equation, which is expressed in terms of absorbance increase in eq 4.

The absobivity constant of the reaction p-nitrophenol ester at 400 nm, A, is obtained by measuring the absorbance at 400 nm of a standard stock solution of p-nitrophenol in 0.1 M NaCl solution. The rate constants are obtained at the experimental pH by measuring the absorbivity constant of p-nitrophenol of 1.138 and a pK for p-nitrophenol of 6.79. Shoos et al. (12) have described the derivation of eqs 4 and 5, and how these equations are used to calculate the Michaelis-Menten kinetic parameters. Fits to log 5 allow calculation of Vmax and Ks in units Abs s⁻¹ and Abs, respectively. In some experiments, the kinetic parameters were calculated by nonlinear-least squares fitting of time-course data. Fits to each of eqs 4 to 6. The interfacial parameters are converted to mole fraction units by using eqs 5; units are in parenthesis.

In eq 4, f is the mole fraction of lipid p-nitrophenyl ester substrate in the mixed micelle and f is the molar analytical concentration of substrate.

The pHs of buffers used for enzyme kinetics and micelle characterization were measured on a Corning Model 115 pH meter equipped with a glass carbon electrode.

Nucleophilic Transferring Experiments - The effects of the nucleophile hydroxide, hydrazide, hydroxylamine, and ethylenediamine on the kinetics of LpL-catalyzed hydrolysis of PNPL were determined by measuring Vmax and Ks for a series of nucleic acid concentrations. Experiments were conducted such that the enzymic rate of nucleic acid transacylation was measured. All solutions for the LpL-catalyzed transacylation, LpL, and substrate, which was formed when hydroxylamine was added to the reaction mixture with other, the other phase was dried over MgSO4, evacuated under a vacuum, and on ethanolic solution of f (7-11). The CMC's for TX100 micelles were measured at 25°C and pH 7.3 in 0.1 M sodium phosphate buffer that contained 0.2 M NaCl, by measuring the shift to longer wavelengths of the absorbance maximum at 526 nm of rhodamine 6G as the analytical concentration of micellar components was increased. The CMC's agree within experimental error, regardless of whether lipid or liquid p-nitrophenyl ester was present. The average CMC for LpL and PNPL were also measured at 25°C and pH 7.3. The CMC of PNPL was found to be 0.05 mM, whereas the CMC of PNPL did not depend on the concentration of rhodamine 6G dye. Therefore, the dye is likely not inducing micellisation of the TX100, lipid p-nitrophenyl ester mixed micelles. Michaelis-Menten constants for LpL-catalyzed hydrolysis of lipid p-nitrophenyl esters were further characterized by gel filtration chromatography on a column of Sepharose CL-6B. As Fig 18 shows, LpL elutes with micelles when the enzyme and TX100 are co-chromatographed. When the chromatographic run is repeated without TX100 LpL is retained by the column and cannot be eluted by the buffer. Therefore, the elution of LpL and TX100 shown in Fig 18 is consistent with LpL associates with the micelles to form a complex that is sufficiently tight to overcome the association of the enzyme with Sepharose CL-6B. Staining of LpL to TX100 micelles does not radically alter the micelles, since the CMC in the presence and absence of LpL as discussed below also support tight complexation of LpL and TX100 micelles.

The kinetics of LpL-catalyzed hydrolysis of micellar liquid p-nitrophenyl esters were changed by the addition of bovine serum albumin, which is a component of the serum. The CMC of the liquid p-nitrophenyl ester in the presence of albumin is increased. Below the CMC the kinetic data are first-order, as the fit eq 2 to timecourses of LpL-catalyzed hydrolysis of PNPL shows. In the section of the Michaelis-Menten equation, as illustrated by the integrated form of the Michaelis-Menten equation, as illustrated by the integrated form of the Michaelis-Menten equation.

Fig 1: Gel filtration chromatography of TX100 micelles. Samples were eluted at 9°C from a 1.15 X 71 cm column of Sepharose CL-6B. Samples were equilibrated in 0.1 M sodium phosphate buffer, pH 6.0, that contained 0.1 M NaCl and were eluted at a flow rate of 7.2 mL h⁻¹. Squares show the elution of PNPL and circles show the elution position of blue dextran (void volume) and p-nitrophenol. Data for reactions conducted above were also determined for LpL-catalyzed hydrolysis of PNPL and PNPP.

RESULTS

CMC's for TX100 micelles were measured at 25°C and pH 7.3 in 0.1 M sodium phosphate buffer that contained 0.2 M NaCl, by measuring the shift to longer wavelengths of the absorbance maximum at 526 nm of rhodamine 6G as the analytical concentration of micellar components was increased. The CMC's agree within experimental error, regardless of whether lipid or liquid p-nitrophenyl ester was present. The average CMC for LpL and PNPL were also measured at 25°C and pH 7.3. The CMC of PNPL was found to be 0.05 mM, whereas the CMC of PNPL did not depend on the concentration of rhodamine 6G dye. Therefore, the dye is likely not inducing micellisation of the TX100, lipid p-nitrophenyl ester mixed micelles. Michaelis-Menten constants for LpL-catalyzed hydrolysis of lipid p-nitrophenyl esters were further characterized by gel filtration chromatography on a column of Sepharose CL-6B. As Fig 18 shows, LpL elutes with micelles when the enzyme and TX100 are co-chromatographed. When the chromatographic run is repeated without TX100 LpL is retained by the column and cannot be eluted by the buffer. Therefore, the elution of LpL and TX100 shown in Fig 18 is consistent with LpL associates with the micelles to form a complex that is sufficiently tight to overcome the association of the enzyme with Sepharose CL-6B. Staining of LpL to TX100 micelles does not radically alter the micelles, since the CMC in the presence and absence of LpL as discussed below also support tight complexation of LpL and TX100 micelles.

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addition, the interfacial kinetic parameters contained in TX100 micelles, to LpL-catalyzed hydrolysis of PNPL. These concentrated manner (data not shown).

p-nitrophenyl esters discussed preparations that contained mole fractions of PNPL characterize the intrinsic fatty acyl specificity of LpL. Table 1 listed in Materials and Methods). Enzyme preparations that contained mole fractions of PNPL lack of product inhibition does not occur. The lack of product inhibition is supported by the linearity of fits to eq 1. In addition, the interfacial kinetic parameters were the same for micelle preparations that contained mole fractions of PNPL. Lack of product inhibition was further confirmed by adding the product lauric acid, contained in TX100 micelles, to LpL-catalyzed hydrolysis of PNPL. These additions had no effect on k.<sup>cat</sup> and k.<sup>cat</sup>/K<sub>m</sub>. The micellar lipoprotein-lipase reaction dynamics have been used to characterize the kinase substrate specificity of LpL. Table 1 contains k.<sup>cat</sup> for PNPA, PNPP, and PNPB. These values are compared to k.<sup>cat</sup> for the water-soluble substrates PNA and PNPB (18,19). Values of k.<sup>cat</sup>/K<sub>m</sub> are also listed in the table.

![Fig. 2: Timecourses for LpL-catalyzed hydrolysis of PNPL contained in TX100 micelles (mean ± SD). Reactions were run at 25.0 ± 0.1°C in 0.03 mL 0.1 M sodium phosphate buffer, pH 7.3, that contained 0.1 mM NaCl.](image)

A. Reaction timecourse when analytical concentration of total micelle components is below the CMC. The reaction contained 0.10 mM TX100, 0.15 mM PNPL, 1.5% glycerol v/v, 10 μg heparin and 3.8 μg LpL. The nonlinear least squares fit to eq 3 of Materials and Methods. Enzyme preparations that contained mole fractions of PNPL, 1.58 glycerol v/v, 10 μg heparin and 7.8 μg LpL. Another weighted least squares fit of the reaction timecourse data to the linear transform of the Michaelis-Menten equation (eq 5 of Materials and Methods). The x-axis label corresponds to the function of absorbance on the left-hand-side of eq 5. The interfacial kinetic parameters calculated from the fit were used to generate the nonlinear fit of the timecourse to eq 4.

LpL-catalyzed hydrolysis of water-soluble acylglycerols that are contained in emulsions or lipoproteins is subject to fatty acid product inhibition when a fatty acid acceptor (albumin or Ce<sup>2+</sup>) is absent from the reaction medium (18-19). The LpL-catalyzed hydrolysis of lipid p-nitrophenyl esters discussed in this paper is unusual in that product inhibition does not occur. The lack of product inhibition is supported by the linearity of fits to eq 1. In addition, the interfacial kinetic parameters were the same for micelle preparations that contained mole fractions of PNPL. Lack of product inhibition was further confirmed by adding the product lauric acid, contained in TX100 micelles, to LpL-catalyzed hydrolysis of PNPL. These additions had no effect on k.<sup>cat</sup> and k.<sup>cat</sup>/K<sub>m</sub>. The micellar lipoprotein-lipase reaction dynamics have been used to characterize the kinase substrate specificity of LpL. Table 1 contains k.<sup>cat</sup> for PNPA, PNPP, and PNPB. These values are compared to k.<sup>cat</sup> for the water-soluble substrates PNA and PNPB (18,19). Values of k.<sup>cat</sup>/K<sub>m</sub> are also listed in the table.

**Table 1: Kinetic Parameters for LpL-Catalyzed Hydrolysis of p-Nitrophenyl Esters**

| Substrate | k.<sup>cat</sup> | k.<sup>cat</sup>/K<sub>m</sub> |
|-----------|----------------|-----------------|
| PNPA<sup>a</sup> | 1.82 | 1.77 ± 10<sup>4</sup> |
| PNPP<sup>a</sup> | 1.22 ± 0.05 | 7.2 ± 0.2 ± 10<sup>4</sup> |
| PNPL<sup>a</sup> | 5.6 ± 0.2 | 6.3 ± 0.3 ± 10<sup>4</sup> |
| PNPB<sup>a</sup> | 2.7 ± 0.4 | 1.9 ± 0.6 ± 10<sup>4</sup> |
| PNPC<sup>a</sup> | 0.6 ± 0.1 | 1.8 ± 0.08 ± 10<sup>4</sup> |

<sup>a</sup> All kinetic runs were done at 25.0 ± 0.1°C and pH 7.1 in 1.0 mL of 0.1 M sodium phosphate buffer that contained 0.1 mM NaCl and 10 μg/mL of heparin. An LpL molecular weight of 15,000 [2] was used to calculate k.<sup>cat</sup>/K<sub>m</sub>. Rate constants for PNPA and PNPP are k.<sup>cat</sup> and k.<sup>cat</sup>/K<sub>m</sub>. Rate constants for PNPA were calculated from data of Quinn et al. (18); rate constants for PNPP were calculated from data of Quinn et al. (19).

<sup>b</sup> For PNPA in 0.05 ± 0.02. The initial mole fraction of substrate was 0.06. The total concentration of micellar components was in the range 0.1–0.4 μM. [LpL] = 2.0 μg/mL.

<sup>c</sup> For PNPP in 0.04 ± 0.01. The initial mole fraction of substrate was 0.064. 0.052 and 0.054. The total concentration of micellar components was in the range 0.4±0.59 μM. [LpL] = 4.2 μg/mL.

The reaction contained 0.5 mM TX100, 1.5% glycerol v/v, 10 μg heparin and 7.8 μg LpL. Another weighted least squares fit of the reaction timecourse data to the linear transform of the Michaelis-Menten equation (eq 5 of Materials and Methods). The x-axis label corresponds to the function of absorbance on the left-hand-side of eq 5. The interfacial kinetic parameters calculated from the fit were used to generate the nonlinear fit of the timecourse to eq 4.

The plots of Figure 3 show that this prediction is correct. Figure 10 is a Lineweaver-Burk plot of the reciprocal form of the data of Figure 3A. A nonlinear-least squares fit to eq 7 yields the following interfacial kinetic parameters: k.<sup>cat</sup> = 0.06 ± 0.01 and V<sub>m</sub> = 5.7 ± 0.8 ± 10<sup>4</sup> s<sup>-1</sup>. Because the micellar concentration (and hence the interface area) is constant in this experiment, the saturation kinetics observed also rules out enzyme binding to the micelle surface as the rate-determining step for PNPL hydrolysis. The reaction rate is plotted in Figure 3B the complete timecourse was also fit to the integrated Michaelis-Menten equation (eq 5, eq 5). The mean values of the interfacial kinetic parameters from these experiments gives k.<sup>cat</sup> = 0.038 ± 0.007 and V<sub>m</sub> = 4.8 ± 0.7 ± 10<sup>4</sup> s<sup>-1</sup>. When one considers that the parameters determined from the initial velocity measurements are effective-
Fig. 3: Initial velocities for LpL-catalyzed hydrolysis of PNPL contained in TX100 micelles.

A. Initial velocities were determined at 25.0 ± 0.1°C in 1.06 mL 0.1 M sodium phosphate buffer, pH 7.37, that contained 0.1 M NaCl, 0.68 µM TX100, 10 µg heparin, 3.4% glycerol v/v, 16.2 µg SPC, and the indicated mole fractions of PNPL. The linear line was generated from the parameters of a nonlinear-least-squares computer fit to eq 7 of the text.

B. Lineweaver-Burk plot of the data of Figure 3A.

Fig. 4: Activation of LpL-catalyzed hydrolysis of PNPL by NH$_2$OH.

A. Fits to eq 1 of timecourse data for reactions at various concentrations of NH$_2$OH. Reactions were done at pH 7.25 and 25.0 ± 0.1°C in 1.06 mL of 0.1 M sodium phosphate buffer that contained 0.1 M NaCl, 0.68 mM TX100, 10 µg heparin, 3.4% glycerol v/v and 16 µg LpL. [NH$_2$OH] = 0 (○), 0.067 M (●) and 0.14 M (□).

B. Dependence of k$_{cat}$ and V$_{max}$ for LpL-catalyzed hydrolysis of PNPL on [NH$_2$OH].

Hydroxylamine increases V$_{max}$ by providing a parallel pathway for turnover of the acylenzyme. The kinetic model for these reaction dynamics will be developed in the DISCUSSION. Nucleophilic trapping by hydroxylamine of a rate-limiting lauryl-LpL intermediate requires that V$_{max}$ activation be accompanied by LpL-catalyzed synthesis of laurylhydroxamate. Laurylhydroxamate formation was verified by TLC after extracting the contents of a spectrophotometer cell (1.05 mL), as described in MATERIALS AND METHODS. The spectrophotometric reaction was scaled up to 7 mL and allowed to proceed for 5 minutes at 25°C in the presence of 29 µg/mL of LpL. A corresponding control contained no enzyme. The reactions were quenched by immersion into an ice bath and extracted with equal volumes of diethyl ether. Quantitation of laurylhydroxamate as described in MATERIALS AND METHODS showed that 30 µg of laurylhydroxamate was formed in the LpL reaction and 13 µg was formed in the control. Scaling up the reaction by another 3-fold resulted in the same ratio of product for the catalyzed and uncatalyzed reactions. Hence, LpL is catalyzing the formation of laurylhydroxamate.