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Aspects on the Catalysis of Lipase from Porcine Pancreas (type VI-s) in Aqueous Media: Development of Ion-pairs

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ABSTRACT

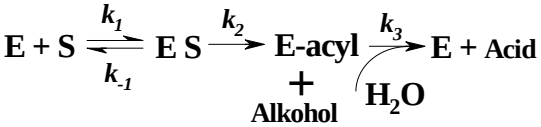
This article reports a first contribution for the elucidation of catalytic mechanism of Lipase from porcine pancreas, type VI-s (PPL), in hydrolyzing an ester substrate in aqueous media. The conclusions were based on the pH-profiles of Michaelis-Menten parameters k_{cat}/K_m, k_{cat} and K_m, as well as on the absolute temperature profile of k_{cat}/K_m, obtained during the hydrolysis of p-nitrophenyl laurate by PPL. It was found that (a) PPL performs catalysis by means of ion pairs formed either as Ser^{152}-O^-/His^{263}-Im^+H and/or Carbonyl-O^-/His^{263}-Im^+H, (b) the parameter k_{cat}/K_m equals to k_1 and thus ES is formed and destroyed in the course of a series of consecutive reactions governed by the dynamic constant $K_S = k_2/k_1$, and (c) the hydrolysis of substrate is assisted by a hydrogen bond developed between deprotonated Asp^{176} and the positively charged imidazole of His^{263} across a pK_a-value 3.85, necessary for efficient catalysis.

Key words: Porcine Pancreas Lipase, mechanism of hydrolysis, *p-nitrophenyl laurate*

INTRODUCTION

Lipases or triacylglycerol acylhydrolases (E.C. 3.1.1.3) catalyze the hydrolysis of various chain-length fatty acids esters and form diacylglyceride or monoacylglyceride, and/or glycerol and free fatty acids. These enzymes have found a variety of industrial applications due to their catalytic properties on a wide spectrum of substrates, as well as due to their high stability towards extreme temperatures and pH-values of the reaction media (Verger 1997; Thomson et al. 1999). The kinetics and mechanisms of lipolysis have been studied to some extend to improve the applications of lipases, as controlled lipolysis is essential for the consistent quality of commercial products (Salleh et al. 2006). The catalytic site of lipases comprises three residues (Asp or Glu, His, Ser) in a straightforward similarity to serine proteases (Derewenda and Sharp, 1993). Generally, the minimum reaction Scheme 1 appears insufficient and ambiguous to explain how Lipase from porcine pancreas type VI-s (PPL) performs catalysis (Jaeger and Eggert, 1994); thus, it seems reasonable to study the catalytic mechanism of PPL.

Scheme 1



This manuscript reports a first contribution for the elucidation of catalytic mechanism of the PPL in hydrolyzing the synthetic ester substrate $CH_3(CH_2)_{10}C(=O)$-ONP (p-nitrophenyl laurate - L-p-ONP) in aqueous buffers of 0.01 M ionic

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strength, containing 0.15% ($^{w}/_{v}$) Arabic gum and 5% ($^{v}/_{v}$) DMSO. For this reason, we analyzed the dependencies of Michaelis-Menten parameters in the hydrolysis of L-p-ONP by PPL as functions of pH-value and/or of k_{cat}/K_m versus absolute temperature, of the reaction means. We found that catalysis is performed by means of ion pairs formed either as Ser^{152}-O^-/His^{263}-Im^+H and/or Carbonyl-O^-/His^{263}-Im^+H, whereas k_{cat}/K_m is almost equal to k_1; furthermore, the hydrolysis of the substrate is assisted by a hydrogen bond developed between Asp^{176} (COO^-) and the positively charged imidazole of His^{263} (lipase numbering), across a pK_a-value 3.85, necessary for efficient catalysis (Theodorou et al. 2007a; 2007b).

MATERIALS AND METHODS

Lipase from porcine pancreas type VI-s (PPL), p-nitrophenyl laurate (L-p-ONP), Arabic gum, 2-mercaptoethanol, dimethylsulfoxide (DMSO) and other chemical were purchased from Sigma.

The pH value of the stock phosphate buffers was checked on a radiometer pH-meter model PHM 82. Suitable pH and temperature activity measurements were carried out in aqueous 0.01 M buffers containing 2 mM 2-mercaptoethanol; same buffers were employed for active site titrations achieved by using the irreversible inhibitor PMSF. The working solutions of the substrate were prepared in DMSO (Theodorou et al, 2007a; 2007b).

All kinetic measurements were performed spectrophotometrically by initial velocities at 405 nm for the L-p-ONP substrate, in a Specord 205 UV-VIS spectrophotometer; aqueous lipase solutions, of about 3930 nM, were prepared containing 5 gr/100 ml Arabic gum. In a typical kinetic run a test-tube was prepared, containing the appropriate quantities of buffer and enzyme solutions, and DMSO at a final total content of 5% ($^{v}/_{v}$); next, a reference-tube was prepared where Arabic gum solution had replaced the enzyme solution. Then, both tubes are placed in an ultrasonic bath for 5 min at the appropriate temperature and the reaction is initiated by the addition of 10-50 μl of substrate solution (in DMSO) in both test and reference tubes. The substrate concentration varied from 10 μM to 200 μM. Again, both tubes are placed in the ultrasonic bath for 15 min; then 1 ml NaOH 0.1 M is added in both tubes and the absorbance of test tube was measured versus the reference one at 405 nm due to the release of p-nitro-phenyl anion ($^{-}O-C_6H_4-NO_2$).

Additional, measurements were performed in the range $5.50 \leq pH \leq 10.50$, in buffers of 0.01 M ionic strength prepared as previously (Theodorou et al. 2007a; 2007b), in order to obtain appropriate pH-(k) profiles. Similar measurements were performed at different temperatures ranging from 13°C to 65°C in phosphate buffers of 0.01 M ionic strength at pH 6.50 (Papamichael et al. 2009).

All parameters were estimated from initial velocities measurements, during the hydrolysis of L-p-ONP substrate by PPL, using nonlinear curve fitting of the appropriate equation to the experimental data. In more details, the experimental data of the dependencies of k_{cat}/K_m k_{cat} and K_m versus pH were best analyzed according to Schemes 2, 3 and 4, and best fitted by different simplified forms of equation (1) comprising three to five hydrogenic forms but only one operative reactive state (Topham et al. 1991). The experimental data of the dependency of k_{cat}/K_m versus temperature were best fitted by equation (2), where $k_{1,0}$, a_0, E_1 and $E_a = E_{-1} - E_2$, represent the values of: rate constant k_1, the ratio k_2/k_{-1}, the activation energies corresponding to the rate constants k_1, k_{-1} and k_2, at the reference temperature $T_0 = 318.15$°K (45°C); T and R are the independent variable (temperature in °K) and the gas constant (8.3144 $Jmol^{-1}$ K^{-1}), respectively (Theodorou et al. 2007a; 2007b; Valasaki et al, 2008; Papamichael et al. 2009; 2010; Papamichael and Theodorou, 2010).

Scheme 2

$$EH_2 \underset{}{\overset{K_{a1}}{\rightleftharpoons}} EH \underset{}{\overset{K_{a2}}{\rightleftharpoons}} E$$

k_{cat}/K_m **or** k_{cat}

Towards Next Step

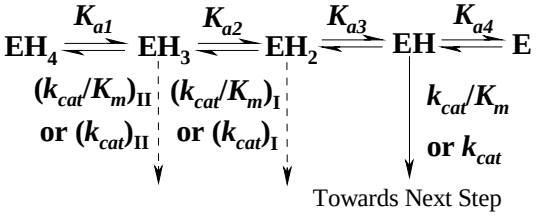
Scheme 3

$$EH_3 \overset{K_{a1}}{\rightleftharpoons} EH_2 \overset{K_{a2}}{\rightleftharpoons} EH \overset{K_{a3}}{\rightleftharpoons} E$$

$(k_{cat}/K_m)_I$ **or** $(k_{cat})_I$; k_{cat}/K_m **or** k_{cat}

Towards Next Step

Scheme 4



$$(k)_{obs} = \sum_{i=1}^{n} \frac{(k)^{lim}_{XH_{i-1}}}{\left(1+\sum_{j=1}^{n} B_{i,j}\right)} \quad (1)$$

$$\frac{k_{cat}}{K_m} = \frac{\alpha_0 e^{\left[\frac{E_a}{R}\left(\frac{1}{T}-\frac{1}{T_0}\right)\right]}}{1+\alpha_0 e^{\left[\frac{E_a}{R}\left(\frac{1}{T}-\frac{1}{T_0}\right)\right]}} (k_1)_0 e^{\left[-\frac{E_1}{R}\left(\frac{1}{T}-\frac{1}{T_0}\right)\right]} \quad (2)$$

Alternative fitting procedures of all series of the above mentioned experimental data were performed also by non-parametric curve fitting methods, and/or by suitable reparametrization of all used simplified forms of equations (1) and (2), until become linear in their parameters, as it has been described previously (Theodorou et al. 2001; Papamichael et al. 2000; Papamichael and Theodorou 2009). In most cases, global minima were approached, and reached to the same results.

RESULTS

The experimental data from the pH-(k_{cat}/K_m) profile are best fitted by equation $(k_{cat}/K_m)_{obs} = (k_{cat}/K_m)^{lim}/(1+10^{pK_{a1}+pK_{a2}+pK_{a3}-3pH}+10^{pK_{a2}+pK_{a3}-2pH}+10^{pK_{a3}-pH}+10^{pH-pK_{a4}})$ corresponding to Scheme 4; the experimental data from the pH-(k_{cat}) and pH-(K_m) profiles are best fitted by equations $(k_{cat})_{obs} = (k_{cat})^{lim}/(1+10^{pK_{a1}+pK_{a2}-2pH}+10^{pK_{a2}-pH}+10^{pH-pK_{a3}})$ and $(K_m)_{obs} = (K_m)^{lim}/(1+10^{pK_{a1}-2pH}+10^{pH-pK_{a2}})$, corresponding to Scheme 3 and 2, respectively.

All pH-profiles were found as bell-shaped showing maxima at almost neutral pH-values in all cases of Michaelis-Menten parameters (Fig. 1); the pK_a-values were estimated with small standard deviations, and are depicted in Table 1.

Table 1 - The estimated parameters from the dependencies of k_{cat}/K_m, k_{ca} and K_m versus pH.

Michaelis-Menten Parameter	pK_a-values
$(k_{cat}/K_m)^{lim}$ = 1424440.43 ± (9.42 × 10^{-8}) (M^{-1} s^{-1})	pK_{a1}= 2.21 ± (2.20 × 10^{-7})
	pK_{a2}= 3.86 ± (1.38 × 10^{-10})
	pK_{a3}= 5.68 ± (6.67 × 10^{-13})
	pK_{a4}= 9.96 ± (1.59 × 10^{-13})
$(k_{cat})^{lim}$ = 125.50 ± (0.13) (s^{-1})	pK_{a1}= 5.85 ± (0.01)
	pK_{a2}= 6.12 ± (4.30 × 10^{-3})
	pK_{a3}= 9.23 ± 2.00 × 10^{-3})
$(K_m)^{lim}$ = 8.18 x 10^{-2} ± (0.01) (mM)	pK_{a1}= 5.84 ± (0.23)
	pK_{a2}= 9.60 ± (0.27)

± (Standard deviations)

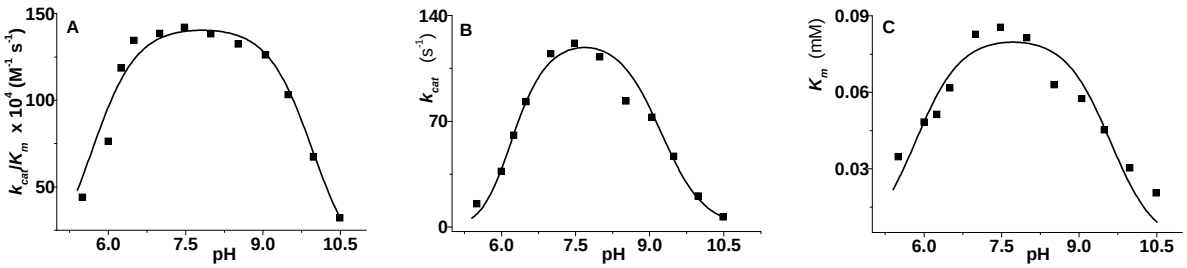


Figure 1 - (A) the pH-(k_{cat}/K_m), (B) the pH-(k_{cat}) and (C) the pH-(K_m) profiles of the hydrolysis of L-p-ONP substrate by PPL. The experimental data are best fitted according to Schemes 4, 3, and 2 and the corresponding equations, respectively.

In the case of absolute Temperature–(k_{cat}/K_m) profile, the experimental data were best fitted by equation (2), and the following parameter values were estimated (Fig. 2):

(a) $\boldsymbol{k_{1,0}}$ **= 1344643.56 ± 4.50 × 10^{-6} M^{-1}s^{-1}**,

which corresponds to the rate constant $\boldsymbol{k_1}$, at 45^{o}C,

(b) $\boldsymbol{a_0 = k_2/k_{-1}}$ **= 21.30 ± (9.80 × 10^{-10})**,

(c) $\boldsymbol{E_a}$ **= 214477.17 ± (2.50 × 10^{-6}) kJ/mol**, and

(d) $\boldsymbol{E_1}$ **= 4315.38 ± (1.4 × 10^{-7}) kJ/mol**.

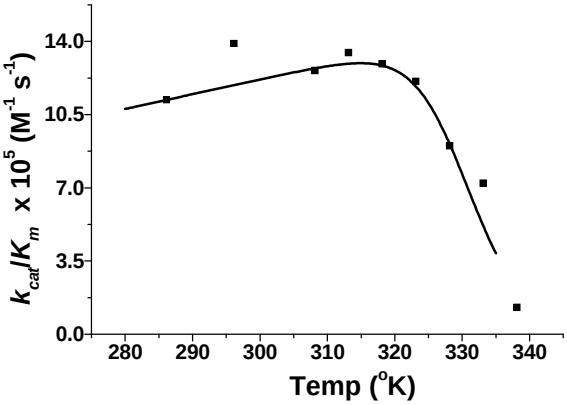


Figure 2 - The dependency of k_{cat}/K_m versus the absolute temperature in the hydrolysis of L-p-ONP substrate by PPL.

DISCUSSION

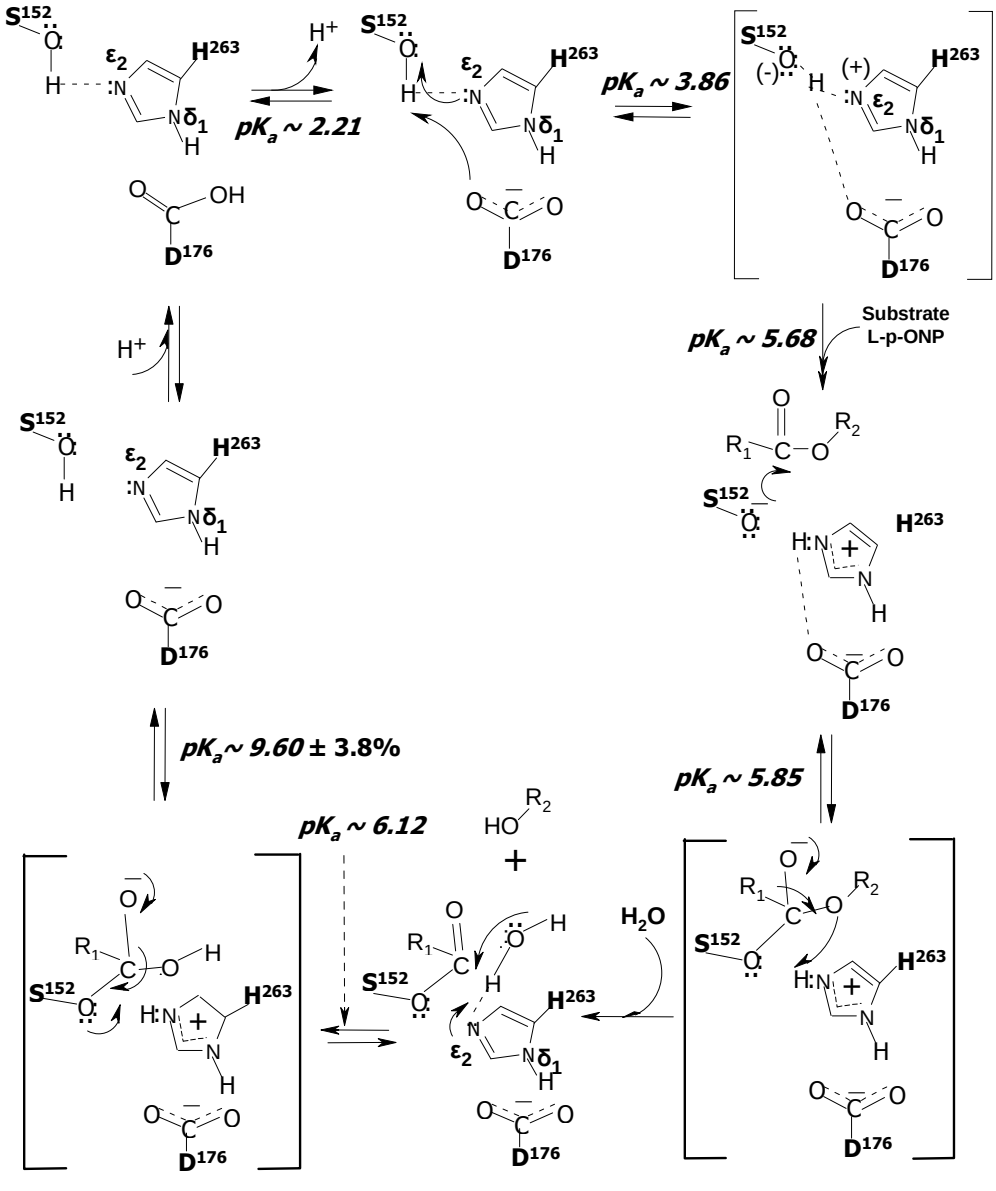
The pH-profiles obtained during the hydrolysis of L-p-ONP by PPL reflect the ionization corresponding to each single Michaelis-Menten parameter, which maintains enzyme active conformation and/or is directly involved in catalysis (Benjamin and Pandey 2000; Rajendran et al. 2009). Similarly, from the absolute temperature–(k_{cat}/K_m) profile were obtained useful relations. However, we should emphasize that both pH and absolute temperature profiles were achieved by performing kinetic measurements in aqueous media of relatively low ionic strength containing 5% ($^{v}/_{v}$) DMSO and 0.15% ($^{w}/_{v}$) Arabic gum, whose usefulness in assays of lipase has been well documented (Mentez and Castro 2005; Salleh et al. 2006).

In all cases, the pK_a-values were estimated with small standard errors. The pH-(k_{cat}/K_m) profile is affected by three ionizable groups in the acidic and one in the basic limb. A value of pK_{a1} = 2.21 could be due to the protonic dissociation of Asp^{176}. Likewise, it seems more likely that a catalytic ion-pair (Ser^{152}-O^{-}/His^{263}-Im^{+}H) is formed across a pK_{a3} = 5.68, while it breaks across a pK_{a4} = 9.96. Furthermore, an estimate of pK_{a2} = 3.86 seems reasonable to be due to the development of a hydrogen bond connecting deprotonated Asp^{176} and positively charged His^{263}. In fact, a reasonable explanation for the development of such a hydrogen bond may be based on the fact that the anion Ser^{152}-O^{-} (PPL numbering) should be free of hydrogen bonds in order to become more nucleophilic when it is attacking on the substrate (Theodorou et al. 2007a; 2007b; Papamichael et al. 2009; 2010; Papamichael and Theodorou, 2010). The best fit of the experimental data of pH-(k_{cat}) and/or pH-(K_m) profiles gave evidence for the estimation of three and/or two pK_a-values, respectively. In these latter cases, it seems more likely that a different ion-pair is formed by means of the carbonyl oxygen of the acyl-group, and the positively charged His^{263} (Carbonyl-O^{-}/His^{263}-Im^{+}H), across almost identical estimates of pK_{a1} = 5.85 and/or 5.84 respectively. This second ion-pair breaks across a pK_a-value 9.60 i.e. the mean value between pK_{a4} = 9.96, pK_{a3} = 9.23 and pK_{a2} = 9.60, estimated from the pH-(k_{cat}/K_m), pH-(k_{cat}) and pH-(K_m) profiles, respectively, which differ only less than 4% (Papamichael et al. 2004; Theodorou et al. 2007a; 2007b; Papamichael and Theodorou, 2010). Finally, an estimated pK_{a2} = 6.12 could denote the protonation of the N$^{\delta 2}$-atom of His^{263} as a H_2O molecule is attacking the acyl-enzyme (Theodorou et al. 2001, 2007a, 2007b; Papamichael et al. 2004, 2009).

From the absolute temperature–(k_{cat}/K_m) profile the ratio $\alpha_0 = k_2/k_{-1} = 21.30$ was obtained, denoting the relation $k_2 >> k_{-1}$ and thus $k_{cat}/K_m \approx k_1$; similarly, the estimated values of activation energies E_a and E_1 are in full agreement to the obtained results from the pH profiles of the Michaelis-Menten parameters. Moreover, the relation $K_S = (k_2 + k_{-1})/k_1 = k_2/k_1$ is valid as $k_2 >> k_{-1}$. On the basis of all mentioned above, it could be concluded that the ES complex is formed and destroyed in the course of a series of consecutive reactions governed by a dynamic constant, the so-called K_S, established according to: $E + S \xrightarrow{k_1} E\,S \xrightarrow{k_2} E\text{-}acyl$.

Additionally, it is not unexpected that under the experimental conditions of the present work it was found that PPL hydrolyses p-nitrophenyl laurate (L-p-ONP) by means of general acid-base catalysis (ionic pairs), and not through a charge-relay-system, as it is the case in serine proteases having similar catalytic site with lipases. Accordingly, a first contribution in the elucidation of the catalytic mechanism of PPL in hydrolyzing the synthetic ester substrate L-p-ONP in aqueous media could be depicted by the below Scheme 5.

Scheme 5



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