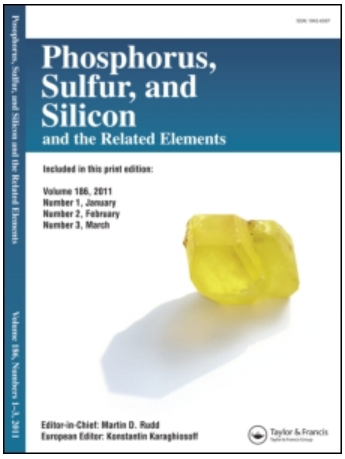

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Phospholipase A<u>1</u> Activity and Catalytic Mechanism of Pancreatic Cholesterol Esterase

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PHOSPHOLIPASE A_1 ACTIVITY AND CATALYTIC MECHANISM OF PANCREATIC CHOLESTEROL ESTERASE

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Abstract The catalytic mechanism of bovine pancreatic cholesterol esterase (CEase) has been probed by using lipid p-nitrophenyl esters and thiophospholipids as substrates. The rate of CEase-catalyzed hydrolyses of p-nitrophenyl esters is highest for substrates that have fatty acyl chains of intermediate length, while solvent isotope effects decrease with increasing chain length. Nucleophilic trapping experiments indicate that k_{cat} for these substrates is rate limited by hydrolysis of acylenzyme intermediates. The k_{cat} for CEase-catalyzed hydrolysis of 1(3)-decanoylthio-2-decanoyl-phosphatidylcholine is nearly the same as that for p-nitrophenyl decanoate, which demonstrates that phospholipolysis is also rate limited by deacylation. Hence, the CEase and serine protease catalytic mechanisms are similar pro forma. This information is used to guide the design of mechanism-based inhibitors, two classes of which, phosphates and enolphosphates, are described herein.

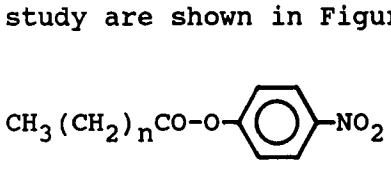
INTRODUCTION

Cholesterol esterase (CEase, EC 3.1.1.13) is an enzyme that is found in various tissues and that catalyzes the hydrolysis of cholesteryl esters, triacylglycerols and phospholipids, as well as the synthesis of cholesteryl esters from cholesterol and fatty acids.[1] Pancreatic CEase is released into the duodenum in response to an alimentary load, and is considered necessary for the absorption of dietary cholesterol into the bloodstream.[2,3] Therefore, an understanding of the mechanism of CEase catalysis should aid in the design of inhibitors that may prove useful in lowering blood cholesterol levels. In this report we describe features of the mechanism of CEase

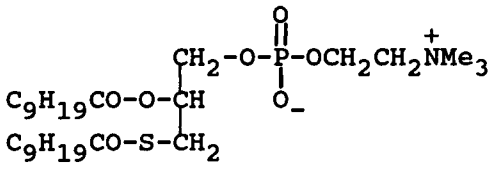
catalysis and characterization of inhibition of the enzyme by compounds that phosphorylate the active site.

RESULTS AND DISCUSSION

The structures of the substrates that were used in this study are shown in Figure 1:



Lipid p-Nitrophenyl Esters (n = 0-10)



1(3)-decanoylthio-2-decanoyl-phosphatidylcholine ($diC_{10}SPC$)

FIGURE 1. Substrates for Cholesterol Esterase

The steady-state kinetic parameters k_{cat} and k_{cat}/K_m and the corresponding solvent isotope effects for CEase-catalyzed hydrolysis of these substrates are gathered in Table 1. Both parameters are maximal for substrates that have fatty acyl chains that are six carbons in length. Addition of the alternate nucleophiles NH_2OH, NH_2NH_2, $MeONH_2$ and imidazole increases k_{cat} and K_m by equal extents when PNPL is the substrate, which is consistent with trapping of a rate-limiting acylenzyme intermediate. Similar experiments by Stout et al.[4] showed that k_{cat} of CEase-catalyzed hydrolysis of PNPB is also rate limited by acylenzyme turnover. Solvent isotope effects are ~2 for k_{cat}/K_m of the phenyl esters with the shortest acyl chains and for k_{cat} of all substates. These isotope effects indicate that the acylation and deacylation transition states are stabilized by proton transfer. Since k_{cat} for hydrolysis of $diC_{10}SPC$ is nearly the same as that for the acyl-similar PNPD, deacylation is also prominently rate limiting for the biomimetic phosphatidylcholine substrate. The large solvent isotope effects for k_{cat} and k_{cat}/K_m of $diC_{10}SPC$ hydrolysis show that CEase stabilizes transition states of lipolysis reactions by proton transfer.

The above-described investigations indicate that the

TABLE I Kinetic Parameters and Solvent Isotope Effects for CEase-Catalyzed Reactions[a]

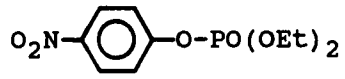
Substrate[b]	k_E, M/s	k_{cat}, s^{-1}	$^{D_2O}k_E$	$^{D_2O}k_{cat}$
PNPA	5.0×10^4	nd[c]	1.81±0.03	nd[c]
PNPB	3.8×10^5	26.9	2.00±0.02	2.36±0.07
PNPH	1.25×10^6	33.1	1.47±0.01	2.01±0.03
PNPO	2.6×10^5	22.3	1.27±0.08	2.6±0.5
PNPD	5.3×10^4	1.5	1.24±0.05	1.65±0.07
PNPL	1.05×10^4	0.7	1.21±0.01	2.0±0.6
$diC_{10}SPC$	2.1×10^3	0.8	3.6±0.3	2.3±0.1

[a]Reactions were run at 25.1 ± 0.4 °C in 0.1 M sodium phosphate buffer, pH 7.01 in H_2O and pD 7.55 in D_2O, that contained 0.1 N NaCl. The parameter k_{cat}/K_m is abbreviated as k_E; $^{D_2O}k_E = k_E^{H_2O}/k_E^{D_2O}$, the solvent deuterium isotope effect on k_{cat}/K_m; similarly $^{D_2O}k_{cat}$ is the solvent isotope effect on k_{cat}.

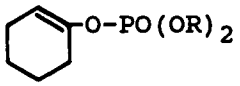
[b]Abbreviations of substrate names: PNPA, p-nitrophenyl acetate (n=0); PNPB, p-nitrophenyl butyrate (n=2); PNPH, p-nitrophenyl hexanoate (n=4); PNPO, p-nitrophenyl octanoate (n=6); PNPD, p-nitrophenyl decanoate (n=8); PNPL, p-nitrophenyl laurate (n=10); $diC_{10}SPC$, 1(3)-decanoylthio-2-decanoylphosphatidylcholine. See FIGURE 1 for the definition of n.

[c]Not determined. The K_m value for PNPA was greater than the solubility of the ester.

catalytic mechanisms of CEase and serine proteases[5-7] are similar, which can be exploited in the design of irreversible inhibitors of CEase. This hypothesis was tested by evaluating the inhibition of porcine pancreatic CEase by phosphate esters (see FIGURE 2 and TABLE II), a class of compounds that are known serine protease inhibitors. The data in TABLE II reveal some structural features that are required for effective inhibition. E600 is an extremely potent CEase inhibitor; 100 nM E600 completely inactivates



Diethyl p-Nitrophenyl Phosphate (E600, **1**)



Dialkylcyclohexenyl Phosphates; R = Et (**2**) or isopropyl (**3**)

FIGURE 2. Irreversible Inhibitors of CEase

TABLE II Second-Order Rate Constants for Irreversible Inhibition of CEase by Phosphate Esters[a]

Inhibitor	k_i, M^{-1} s^{-1}
Diethyl p-nitrophenyl phosphate, **1**	1.03 ± 0.07 x 10^5
Diethylcyclohexenyl phosphate, **2**	74 ± 3
Diisopropylcyclohexenyl phosphate, **3**	7.6 ± 0.3

[a]Inhibition reactions were assayed at 25.0 ± 0.2 °C in 0.1 M sodium phosphate buffer, 0.1 N NaCl, 2-3% MeCN (v/v). Other conditions: **1**, pH 7.31; **2**, pH 7.02, 1 mM sodium taurocholate; **3**, pH 6.94, 1 mM sodium taurocholate

the enzyme in 10 minutes. The enolphosphates are less potent irreversible inhibitors, and CEase shows a ten-fold preference for the enolphosphate that has the less bulky R groups. These data are currently being used to guide the design of biomimetic phosphorylating inhibitors of CEase in our laboratory.

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