Vol. 134, No. 1, 1986 BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS
January 14, 1986 Pages 386-392

PHENYL-n-BUTYLBORINIC ACID IS A POTENT TRANSITION STATE ANALOG INHIBITOR OF LIPOLYTIC ENZYMES

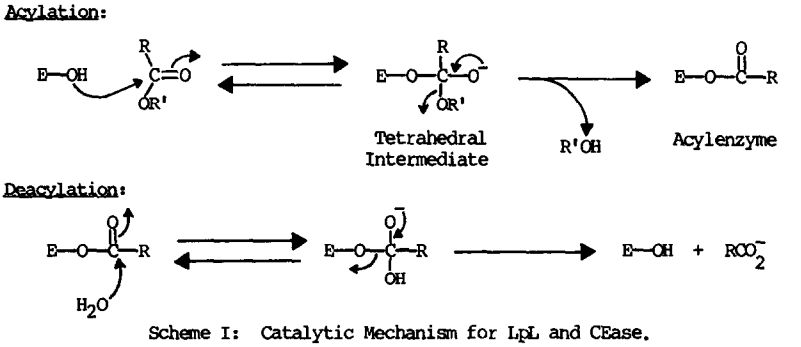
Larry D. Sutton, Jay S. Stout, Lynn Hosie,
Patricia S. Spencer and Daniel M. Quinn

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

Received November 27, 1985

Summary: The cholesterol esterase and lipoprotein lipase catalyzed hydrolyses of the water-soluble substrate p-nitrophenyl butyrate are competitively inhibited by butaneboronic acid and phenylboronic acid. Phenyl-n-butylborinic acid has been synthesized and characterized as an ultrapotent transition state analog inhibitor: K_i = 2.9 ± 0.6 nM and 1.7 ± 0.3 μM for the cholesterol esterase and lipoprotein lipase reactions, respectively. These results are interpreted in terms of transition state structure and stabilization.

Lipoprotein lipase (LpL) catalyzes the hydrolysis of triacylglycerols and phospholipids of triacylglycerol-rich lipoproteins in the bloodstream [1]. Cholesterol esterase (CEase) catalyzes the hydrolysis of emulsified fats, including cholesteryl esters [2], and is necessary for absorption of cholesterol through the intestinal mucosa [3,4]. Both enzymes catalyze the hydrolysis of the water-soluble substrate p-nitrophenyl butyrate (PNPB). Catalysis by these enzymes probably occurs via an acylenzyme mechanism [5-9], which is outlined in Scheme I:



Scheme I: Catalytic Mechanism for LpL and CEase.

Abbreviations: LpL, lipoprotein lipase; CEase, cholesterol esterase; PNPB, p-nitrophenyl butyrate; BBA, butaneboronic acid; PBA, phenylboronic acid; PBBA, phenyl-n-butylborinic acid.

0006-291X/86 $1.50

In the scheme E-OH represents the active site serine whose γ-OH group nucleophilically attacks the carbonyl carbon of the scissile bond of the substrate. The mechanism involves tetrahedral intermediates in both the acylation and deacylation stages and is similar in many respects to the serine protease mechanism [10-12]. Solvent isotope effect experiments suggest that proton transfers are catalytic elements of LpL [5] and CEase [9] reaction dynamics. An important prediction based on the mechanism is that compounds that bind to the active site in a manner that mimics the structure of the tetrahedral intermediate(s) should be potent transition state analog inhibitors [13].

Judiciously designed boronic acids are transition state analog inhibitors of serine proteases [13-16]. Butaneboronic acid (BBA) and phenylboronic acid (PBA) were used herein to probe the mechanism and inhibition of LpL and CEase and were found to be good competitive inhibitors. Phenyl-n-butylborinic acid (PBBA), which contains the hydrocarbon functionalities of both above-mentioned boronic acids and retains the electrophilic boron, was synthesized and utilized as a probe of lipolytic enzyme mechanism and inhibition.

MATERIALS AND METHODS

Enzymes: Porcine pancreatic CEase (carboxylic ester hydrolase, EC 8.1.1.1) was purified by a procedure developed in Brockman's laboratory (Brockman, H.L., personal communication). The detailed procedure will be described by Brockman in a separate publication. A brief description follows: 1) homogenize and blend 400 grams of frozen pancreas; 2) remove fat and tissue debris by centrifugation at 13000 x g; 3) batch steps, including 10% saturated ammonium sulfate precipitation of other proteins, heat precipitation of other proteins at 53-54 ^{o}C, 55% saturated ammonium sulfate precipitation of CEase, and t-butanol extraction of CEase; 4) gel filtration on a 5x60 cm column of Sephacryl S-200; 5) chromatography on a 2.5x30 cm column of PBE-94; 6) chromatography on a 1.5x30 cm column of PBE-94; 7) gel filtration on a 2x100 cm column of Sephadex G-100.

Bovine milk LpL (EC 3.1.1.34) was purified by affinity chromatography on heparin-Sepharose [17-19].

Other Materials: Sodium taurocholate, heparin from porcine intestinal mucosa, Sephadex G-100, PNPB, BBA and PBA were purchased from Sigma Chemical Co. Phenyllithium, butyllithium and tri-n-butylborate were purchased from Aldrich. Porcine pancreas was obtained during slaughter from a local slaugterhouse and immediately frozen on dry ice. Fresh bovine milk was obtained from a local dairy farm. Sephacryl S-200 and PBE-94 (polybuffer exchanger for chromatofocusing) were purchased from Pharmacia Fine Chemicals. Solvents, buffer components and salts were commercially available reagent grade chemicals.

PBBA was synthesized by reacting tri-n-butylborate with butyllithium and phenyllithium in toluene at -70 ^{o}C under a nitrogen atmosphere, and was purified by recrystallization of the ethanolamine adduct from toluene [20,21].

The melting point of the purified adduct is 104.5-105.5 °C (lit. m.p. 108 °C [21]). NMR and mass spectra are consistent with the structure of the adduct.

Reaction Kinetics and Data Reduction: Buffers for enzyme reactions and reaction conditions are described in Table I. PNPB and PBBA-ethanolamine adduct were dissolved in acetonitrile and injected into the reaction buffer. The PBBA adduct rapidly hydrolyses to PBBA after injection. BBA, PBA and the enzymes were dissolved in buffer and injected into reaction solutions. The reactions were monitored by following the formation of p-nitrophenoxide at 400 nm on Beckman DU40 or DU7 UV-visible spectrophotometers that are interfaced to IBM Personal Computers.

The timecourses for CEase-catalyzed hydrolysis of PNPB are described by the integrated form of the Michaelis-Menten equation when $[S]_o \geq K_m$ [9]:

$$t = \frac{K_m}{V_{max}} \ln \frac{A_\infty - A_o}{A_\infty - A} + \frac{1}{V_{max}\,\Delta\varepsilon} (A - A_o) \qquad (1)$$

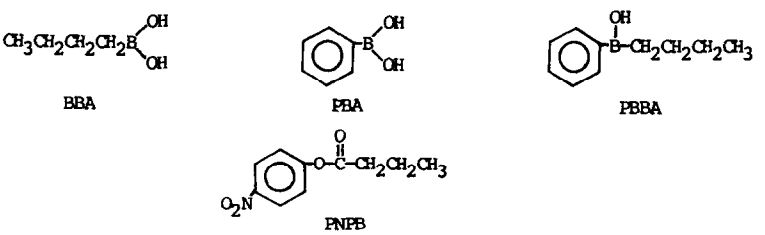
K_m and V_{max} were calculated by fitting absorbance timecourse data to eq 1 by nonlinear least squares procedures. When $[S]_o << K_m$, timecourse data were fit by nonlinear least squares to a first-order kinetics function, as described by Stout et al. [9]. Initial velocities were calculated by linear-least squares analysis of $\leq$ 5% of the respective timecourses. Fits to the Lineweaver Burk equation and fits of Dixon plots [22] were calculated by weighted linear least squares [23,24]. The second-order rate constant for spontaneous hydrolysis of PNPB at 25 °C and pH 7.01 was determined by dividing the intercept of a plot of initial velocities versus sodium phosphate buffer concentration by $[PNPB]_o$ and by 55.5 M, the concentration of water.

RESULTS

Scheme II shows the structures of the inhibitors and, for comparison, that of the substrate PNPB. The kinetic parameters for LpL and CEase-catalyzed hydrolyses of PNPB and the inhibition constants for the inhibitors of Scheme II are given in Table I.

DISCUSSION

Enzymes catalyze their reactions by stabilizing the transition states, thereby lowering the energy of activation [13,27]. One can estimate the



Scheme II: Substrate and Inhibitors for LpL and CEase.

Table I: Kinetic and Inhibition Constants for LpL and CEase Reactions

Enzyme	Compound	K_m, mM	$\frac{k_{cat}}{K_m}$	K_i
LpL[a]	PNPB[b]	0.78 ± 0.02	5.3 ± 0.2 x 10^3 M^{-1} s^{-1}	-
	PBA[b]	-	-	6.9 ± 0.4 μM
	PBBA	-	-	1.7 ± 0.3 μM
CEase[c]	PNPB	0.15 ± 0.02	2.0 ± 0.2 x 10^5 M^{-1} s^{-1}	-
	BBA	-	-	0.23 ± 0.01 mM
	PBA	-	-	36 ± 3 μM
	PBBA	-	-	2.9 ± 0.6 nM

[a]LpL reactions were monitored by measuring initial velocities at 25.0 ± 0.1 °C in 1.00 ml of 0.1 M sodium phosphate buffer, pH 7.01, that contained 0.1 N NaCl, 10 μg heparin, 2% v/v MeCN and varying concentrations of PNPB and inhibitors. K_i's were calculated from the intersection of Dixon plots at two concentrations of PNPB.

[b]Kinetic parameters for PNPB were calculated from data in reference 5, using LpL molecular weight of 41700 [25]. K_i for PBA is from the same reference.

[c]CEase reactions were run under the conditions of footnote a, except that the the buffer contained 1 mM sodium taurocholate and no heparin. K_i's for BBA and PBA were calculated from the intersection of Dixon plots at two concentrations of PNPB. K_i for PBBA was calculated by Henderson tight binding analysis [26]. Kinetic parameters for PNPB hydrolysis were calculated by fitting timecourse data to eq 1.

stabilization of the enzymic transition state relative to a particular standard reaction transition state by calculating the free energy of transition state binding according to eq 2 [27]:

$$\Delta G_b^* = -RT \ln (k_E/k_{SR}) \quad (2)$$

In this equation $k_E = k_{cat}/K_m$, the second-order rate constant for LpL or CEase catalyzed hydrolysis of PNPB. The rate constant k_{SR} is that for the nonenzymic standard reaction, spontaneous hydrolysis of PNPB, for which k_{SR} = 2.6 x 10^{-8} M^{-1} s^{-1}. The catalytic accelerations and transition state binding energies for LpL and CEase-catalyzed hydrolyses of PNPB are given in Table II.

A transition state analog inhibitor binds to an enzyme in a mode that resembles the catalytic transition state(s), thereby eliciting interactions with the enzyme that are involved in transition state stabilization [13]. Boronic and borinic acids bind to serine enzymes via nucleophilic interaction

Table II: Transition State and Transition State Analog Binding Energies

Enzyme	Compound	k_E/k_{SR}	ΔG_b^*, kcal mol^{-1}	K_m/K_i	ΔG_b, kcal mol^{-1}
CEase	PNPB	7.7×10^{12}	-17.6	-	-
	PBBA	-	-	52000	-6.4
LpL	PNPB	2.0×10^{11}	-15.4	-	-
	PBA	-	-	110	-2.8
	PBBA	-	-	460	-3.6

of the γ-OH of the active site serine with the electrophilic boron to produce adducts that resemble tetrahedral intermediates of the catalytic cycle [13-16] (cf. Scheme I). Should tight binding of tetrahedral intermediates be a prominent source of the catalytic power of LpL and CEase, one would expect boronic or borinic acids to be potent inhibitors of the enzymes. The data of Tables I and II demonstrate that this is so. BBA and PBA bind only about as tightly to CEase as does PNPB. The borinic acid PBBA was synthesized in the hope that the binding determinants of BBA and PBA with CEase could be combined in the same molecule, thus giving a K_i of ∿8 nM, the product of the K_i's for BBA and PBA. The K_i = 2.9 nM of Table I is better than expected. In fact, PBBA is by far the most potent inhibitor to date of a lipolytic enzyme. PBBA binds to CEase at least 52000-fold[1] more tightly than does PNPB. PBBA binds to LpL 760-fold more tightly than does PNPB and 4-fold more tightly than does PBA (5,28), and therefore is the most potent LpL inhibitor to date.

The free energy of transition state analog binding, relative to that of PNPB, can be calculated by using an equation that is analogous to eq 2 (13):

$$\Delta G_b = -RT \ln (K_m/K_i) \quad (3)$$

[1] K_m overestimates the affinity of PNPB and CEase by at least an order of magnitude because deacylation is the rate-determining step monitored by V_{max} [9]. In this case, $K_m = K_s k_3/k_2$, where k_2 and k_3 are the rate constants for acylation and deacylation, respectively, and K_s is the dissociation constant of the Michaelis complex. Hence, PBBA binds at least 520,000-fold more tightly to CEase than does the structurally analogous substrate PNPB.

Inhibitor/substrate binding ratios and free energies of inhibitor binding are also given in Table II. The catalytic accelerations, k_E/k_{SR}, of Table II show that LpL and CEase are powerful catalysts for PNPB hydrolysis. Moreover, the potency of inhibition of CEase and LpL by PBBA parallels the respective catalytic accelerations, which indicates that kinetic studies of substrate turnover can be useful in the rational design of potent mechanism-based inhibitors of lipolytic enzymes. For LpL and CEase the PBBA binding free energy calculated from eq 3 is 23% and 36%, respectively, of the transition state binding energy calculated from eq 2. Therefore, a prominent source of the catalytic power of LpL and CEase is stabilization of tetrahedral intermediates, as well as of the transition states for formation and decomposition of these intermediates.

ACKNOWLEDGEMENTS: This work was supported by American Heart Association Grant-In-Aid 84-1003 and by a U.S. Public Health Service New Investigator Research Award (HL30089) and a Research Career Development Award (HL10583) to DMQ.

REFERENCES

1. Quinn, D.M., Shirai, K., and Jackson, R.L. (1983) Prog. Lipid Res. 22, 35-78.
2. Rudd, E.A., and Brockman, H.L. (1984) in Lipases (Burgstrom, B., and Brockman, H.L., eds.), pp. 185-204, Elsevier, Amsterdam.
3. Bhat, S.G., and Brockman, H.L. (1982) Biochem. Biophys. Res. Commun. 109, 486-492.
4. Gallo, L.L., Clark, S.B., Myers, S., and Vahouny, G.V. (1984) J. Lipid Res. 25, 604-612.
5. Quinn, D.M. (1985) Biochemistry 24, 3144-3149.
6. Kinnunen, P.K.J., Virtanen, J.A., and Vainio, P. (1983) Atherosclerosis Rev. 11, 65-105.
7. Lombardo, D., and Guy, O. (1981) Biochim. Biophys. Acta 657, 425-437.
8. Lombardo, D. (1982) Biochim. Biophys. Acta 700, 67-74.
9. Stout, J.S., Sutton, L.D., and Quinn, D.M. (1985) Biochim. Biophys. Acta, in press.
10. Stroud, R.M. (1974) Sci. Am. 231, 74-88.
11. Blow, D.M. (1976) Acc. Chem. Res. 9, 145-152.
12. Hegazi, M.F., Quinn, D.M., and Schowen, R.L. (1978) in Transition States of Biochemical Processes (Gandour, R.D., and Schowen, R.L., eds.), pp. 355-428, Plenum Press, New York.
13. Wolfenden, R. (1978) in reference 12, pp. 555-578.
14. Kettner, C.A., and Shenvi, A.B. (1984) J. Biol. Chem. 259, 15106-15114.
15. Koehler, K.A., and Lienhard, G.E. (1971) Biochemistry 10, 2477-2483.
16. Mathews, D.A., Alden, R.A., Birktoft, J.J., Freer, S.T., and Kraut, J. (1975) J. Biol. Chem. 250, 7120-7126.
17. Iverius, P.H. (1971) Biochem. J. 124, 677-683.
18. Kinnunen, P.K.J. (1977) Med. Biol. 55, 187-191.

19. Matsuoka, N., Shirai, K., and Jackson, R.L. (1980) Biochim. Biophys. Acta 620, 308-316.
20. Letsinger, R.L., and Skoog, I. (1955) J. Amer. Chem. Soc. 77, 2491-2494.
21. Letsinger, R.L., and Nazy, J.R. (1958) J. Org. Chem. 23, 914-915.
22. Segel, I.H. (1975) Enzyme Kinetics, pp. 109-111, John Wiley & Sons, New York.
23. Cleland, W.W. (1967) Adv. Enzymol. Relat. Areas of Mol. Biol. 29, 1-32.
24. Quinn, D.M., Shirai, K., Jackson, R.L., and Harmony, J.A.K. (1982) Biochemistry 21, 6872-6879.
25. Olivecrona, T., Bengtsson, G., and Osborne, J., Jr. (1982) Eur. J. Biochem. 124, 629-633.
26. Reference 22, pp. 158-159.
27. Schowen, R.L. (1978) in reference 12, pp. 77-117.
28. Vainio, P., Virtanen, J.A., and Kinnunen, P.K.J. (1982) Biochim. Biophys. Acta 711, 386-390.