p-Nitrophenyl and Cholesteryl-N-Alkyl Carbamates as Inhibitors of Cholesterol Esterase*

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Recently there has been increased interest in pancreatic cholesterol esterase (CEase) due to an observed correlation between enzymic activity in vivo and absorption of dietary cholesterol (1, 2). Gallo et al. (2) have shown that removal of the enzyme from pancreatic juice results in an 80% reduction in the uptake of cholesterol into the bloodstream in rats. Since the association between serum cholesterol levels and atherosclerosis has been well documented (3), an investigation into the mechanism of action of this enzyme may lead to the design of mechanism-based inhibitors which could be of future therapeutic use.

The enzyme is not specific for cholesterol esters. It will catalyze the hydrolysis of acylglycerols and phospholipids at the micellar interface (4, 5) and will also hydrolyze esters of p-nitrophenol in the aqueous or micellar phase (6–8). Nuclear magnetic resonance studies on the porcine and human CEase-catalyzed hydrolyses of p-nitrophenyl esters (8, 9), and kinetic studies of cholesterol exchange into cholesteryl oleate (9), are consistent with the formation of an acylenzyme intermediate. Solvent deuterium kinetic isotope effects on the turnover of p-nitrophenyl butyrate (PNPB) by the porcine enzyme indicate that both the acylation and deacylation steps involve proton transfer (8). In addition, chemical modification experiments suggest that serine and histidine are essential for hydrolysis of both water and micellar-soluble substrates (10, 11), which indicates that these residues are located in the active site and not at positions on the enzyme that are responsible for the binding to the interface (11). The mechanism of action of CEase therefore resembles that of the serine proteases (8–12).

Therefore, one would predict that compounds containing a carbamate functionaility will be good inhibitors of CEase, since such compounds have been shown to be good inhibitors of many serine hydrolases (13, 14). In this paper, we report inhibition of CEase by the p-nitrophenyl and cholesteryl-N-alkyl carbamates that are shown in Scheme I. Our results for the p-nitrophenyl derivatives are interpreted in terms of a carbamylenzyme mechanism, which is analogous to an acylenzyme mechanism and therefore substantiates the results of earlier work (8, 9).

EXPERIMENTAL PROCEDURES

Materials—Porcine pancreatic CEase (carboxylic-ester hydrolase, EC 3.1.1.13) was isolated according to a procedure developed in Dr. H. Brockman's laboratory, as outlined by Stout et al. (8). Bovine pancreatic CEase, chymotrypsin, trypsin, sodium taurocholate, egg phosphatidylcholine (PC), PNPB, p-nitrophenyl chloroformate, cholesteryl chloroformate, phenylboronic acid (PBA) and n-octylamine were purchased from Sigma. All other materials were commercially available reagent grade products.

Synthesis of Carbamates 1–4—Literature methods for the synthesis of carbamate inhibitors shown in Scheme I (14, 15) were modified slightly. The respective chloroformates (0.01 mol) were dissolved in dry dichloromethane (15 ml) to which was added, over a period of 1 h, a solution of the amine (0.016 mol) in dichloromethane (15 ml). The solutions were stirred overnight. p-Nitrophenyl-N-butyl carbamate (compound 1 of Scheme I) and p-nitrophenyl-N-octyl-carbamate (compound 2 of Scheme I) were extracted as follows: the dichloromethane layer was dried, filtered, and evaporated to dryness. The residue was then chromatographed on silica gel, which was developed by elution with one of the following solvents: dichloromethane, dichloromethane-methanol, or 2-propanol. The fractions containing the p-nitrophenyl ester were combined and evaporated to dryness. The residue was then triturated with ether to give the pure p-nitrophenyl ester.

Experimental procedures are outlined by Stout et al. (8). Bovine pancreatic CEase, chymotrypsin, trypsin, sodium taurocholate, egg phosphatidylcholine (PC), PNPB, p-nitrophenyl chloroformate, cholesteryl chloroformate, phenylboronic acid (PBA) and n-octylamine were purchased from Sigma. All other materials were commercially available reagent grade products.

Abbreviations: CEase, cholesterol esterase; PNPB, p-nitrophenyl butyrate; PBA, phenylboronic acid; PC, phosphatidylcholine; TC/PC micelles, mixed micelles of taurocholate and phosphatidylcholine; TLC, thin layer chromatography.

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1 Abbreviations: CEase, cholesterol esterase; PNPB, p-nitrophenyl butyrate; PBA, phenylboronic acid; PC, phosphatidylcholine; TC/PC micelles, mixed micelles of taurocholate and phosphatidylcholine; TLC, thin layer chromatography.

2 CEase catalyzes the hydrolysis of lipid p-nitrophenyl esters that are contained in mixed micelles with Triton X-100, J. S. Stout and D. M. Quinn, unpublished observations.
Cholesterol Esterase Inhibition

In Equation 1, \( \%A \) is the % activity remaining at various times, \( \%A = (\Delta %A)e^{-k_{oe}} + \%A_{o} \) is the difference between the % activity at time 0 and at infinite reaction time, and \( \%A_{o} \) is the % activity remaining at infinite reaction time. At high concentrations of inhibitors, the changes in absorbance due to PNDB hydrolysis were small, and the control reaction over the time range was linear. Therefore, the apparent first-order rate constant was calculated by fitting the progress curve directly by nonlinear least squares.

Inhibition Studies with Compounds 1 and 2—The CEase-catalyzed hydrolysis of PNDB was followed continuously at 400 nm, in the presence and absence of inhibitor. A typical run was as follows: aliquots of acetonitrile solutions of substrate and inhibitor (to give final concentrations in the cell of \( 47 \mu M <(K_{i}) \) and 0–5 \( \mu M \), respectively) were added to buffer that had been equilibrated at 25.0 ± 0.1°C. The concentration of acetonitrile was kept constant at 2% (v/v). Enzyme was added to initiate the reaction. A typical CEase concentration in the cell was 0.3 \( \mu g/ml \) (3.4 nm). Inactivation in the presence of TC/PC micelles (1.87 mM) was performed in a similar manner. Duplicate or triplicate sets of data were collected for each inhibitor concentration.

Inhibition Studies with Compounds 3 and 4—CEase (3.4 nm) was incubated for 1 min with the micellar solutions of compounds 3 and 4 (4.5 and 3 mol % in 1.87 mM TC/PC, respectively), or with micelles only, prior to the addition of substrate (25 \( \mu M \)). Return of Activity and Protection by Phenylboronic Acid—CEase (14 nm) was incubated with compound 1 or 2 (1 and 0.25 \( \mu M \), respectively) in the absence and presence of PBA (0.15 or 1.2 mM), a known competitive inhibitor of CEase (17). After a period of 9 min to allow for 90% inhibition by compound 1 or 2, the enzyme solution was diluted into buffer or buffer containing TC/PC micelles (1.87 mM). Aliquots of the diluted enzyme/inhibitor solutions were taken at time intervals and tested for activity with PNDB (0.2 mM). Corresponding controls were performed in the absence of inhibitor to correct for spontaneous loss in enzyme activity. The initial rates were calculated using a linear least squares program and the first-order rate constants for the return of activity were calculated by linear least squares analysis of plots of \( \log( (A_{t} - A)/A_{o} - A_{t}) \) against time. Turnover of the cholesteryl carbamates by CEase was analyzed by TLC. Enzyme (12 nm) was incubated in a volume of 0.2 ml with compound 3 or 4 (4.5 or 3 mol %, respectively) in TC/PC micelles (1.87 mM) for 1 h at 25.0 ± 0.1°C. Hexane (1 ml) was added and the mixture stirred. The standards were treated in a similar fashion. The hexane phase was spotted on a precoated plastic silica TLC slide (Macherey-Nagel), and the slide was developed with dichloromethane/methanol (98:2 v/v).

Nucleophilic Activation of the Rate of Return of Activity—CEase (0.3 nm) was incubated with compound 1 (1 \( \mu M \)) for 9 min, and the reaction mixture was diluted 2.5-fold into a solution of hydroxyamine (50 mM, pH 7.00). The return of activity was measured by taking aliquots of the hydroxylamine solution and diluting into buffer that contained PNDB (0.1 mM); initial rates were calculated by linear least squares and the % of activity was calculated by dividing by the initial rates measured from a corresponding control that had not been inhibited by compound 1. The effect of hydroxylamine on the nonenzymatic hydrolysis of compound 1 was measured. Initial rates of the nonenzymic hydrolysis of compound 1 (0.6 mM) were measured at 400 nm and at 25.0 ± 0.1°C in the presence and absence of hydroxyamine (0.05 mM) in 1.0 mM sodium phosphate buffer, pH 7.00, that contained 0.1 N NaCl.

RESULTS

p-Nitrophenyl-N-alkyl Carbamates 1 and 2—Compounds 1 and 2 show time-dependent, pseudo-first-order inhibition of both bovine and porcine pancreatic CEase. Fig. 1B shows the decrease in activity with time of the porcine enzyme in the presence of carbamate 1. The mechanism for inhibition in the presence of substrate is shown in Scheme I. Since the inhibition of CEase follows first-order kinetics over the observed time period, the rate of hydrolysis of \( EI' \) must be significantly slower than the rate of formation of \( EI' \) (13). Therefore,  

The molarity of the enzyme is calculated using a molecular weight of 90,000, as determined by sodium dodecyl sulfate-gel electrophoresis.
Fig. 1. Inhibition of the CEase-catalyzed hydrolysis of PNPB by p-nitrophenyl-N-butyl carbamate 1. A, timecourse for the CEase-catalyzed hydrolysis of PNPB (47 μM) in the presence (●) and absence (▲) of 1 (1 μM). The concentration of CEase was 0.1 mM. Reactions were run at 25.0 ± 0.1 °C in 0.1 M sodium phosphate buffer, pH 7.00, that contained 0.1 M NaCl. B, exponential decrease in activity with time of CEase in the presence (●) and absence (▲) of TC/PC micelles. Calculations of % activities and first-order rate constants are described under “Experimental Procedures.”

Nonlinear least squares fitting to Equation 2 of the text gave kapp = 0.63 ± 0.09 μM and k2 = 2.31 ± 0.07 × 10−2 s−1.

values of K1 and k2 can be calculated from Equation 2 (18):

\[
 k_{\text{app}} = \frac{k_2 [I]}{K_1^*(S) + K_m}
\]

(2)

Fig. 2 shows the plot of \( k_{\text{app}} \) against \([I]\) for the inhibition of carbamate 1. \( K_1^*(1 + [S]/K_m) \) and \( k_2 \) were calculated by fitting the data to Equation 2 by nonlinear least squares. \( K_1^* \) was calculated by dividing by the factor (1 + [S]/K_m), where [S] = 47 μM and K_m = 65 ± 21 μM, to give \( k_1^* = 0.63 ± 0.09 \mu M \). The values of \( k_2 \) and the bimolecular rate constant, \( k_2/K_1^* \), are

**Table I**

Second-order rate constants for the inactivation of CEase by p-nitrophenyl carbamates

<table>
<thead>
<tr>
<th>Inhibitor</th>
<th>No micelles*</th>
<th>TC/PC micelles*</th>
</tr>
</thead>
<tbody>
<tr>
<td>N-butyl (compound 1)</td>
<td>1.01 ± 0.01</td>
<td>1.7 ± 0.1</td>
</tr>
<tr>
<td>N-octyl (compound 2)</td>
<td>48 ± 3</td>
<td>2.70 ± 0.06</td>
</tr>
</tbody>
</table>

*Kinetic runs were done at 25.0 ± 0.1°C and pH 7.00 in 0.1 M sodium phosphate buffer that contained 0.1 M NaCl. The concentrations of compounds 1 and 2 were 0.6 and 0.049 μM, respectively. First-order rate constants were calculated as described under “Experimental Procedures” and in the legend of Fig. 1. The second-order rate constant for I was calculated by dividing the first-order rate constant by \([I]\).

Reactions were conducted as described in the previous footnote, except that the reaction mixture contained 1.87 mM TC/PC micelles and 1.4 μM compound 1 or 1.0 μM compound 2.

2.3 ± 0.1 × 10−2 s−1 and 3.7 ± 0.5 × 104 M−1 s−1, respectively.

Table I gives a comparison of the second-order rate constants for the inhibition of CEase by carbamates 1 and 2, which shows that the inactivation by carbamate 2 is more effective than by carbamate 1 by a factor of 48. Table I also gives a comparison of the second-order rate constants for the inhibition by carbamates 1 and 2 in the absence and presence of TC/PC micelles. The inhibition by carbamate 1 is facilitated by almost 2-fold in the presence of micelles, whereas inhibition by compound 2 is impeded by almost 20-fold. However, the latter compound is still more effective than compound 1 in inhibiting CEase at the lipid-water interface.

In the presence of 0.15 mM of the competitive inhibitor PBA, compounds 1 and 2 (1 and 0.1 μM, respectively) afford only 50% inactivation of the porcine enzyme over a period of 1 h, in comparison to a 90–95% loss in activity observed in the absence of PBA. An increase in the concentration of PBA to 1.2 mM results in a loss of only 20% of the CEase activity effected by compound 1 over a period of 1 h.

Incubation of CEase with either compound 1 or 2 does not give complete inhibition, since 5–10% residual activity remains after prolonged incubation and with increasing inhibitor concentration. Assay of CEase activity on dilution of the inhibited enzyme into buffer that contains PNPB (0.2 mM) shows a gradual return of activity with time. First-order rate constants for return of activity of CEase after inhibition by compounds 1 and 2 are given in Table II. The presence of TC/PC micelles resulted in a rate of the return of activity of CEase after inhibition by compound 1, but has no effect, within experimental error, on the reactivation of the enzyme.

**Table II**

First-order rate constants for return of activity of CEase after inactivation by compounds 1 and 2

<table>
<thead>
<tr>
<th>Inhibitor</th>
<th>TC/PC micelles</th>
</tr>
</thead>
<tbody>
<tr>
<td>N-butyl (compound 1)</td>
<td>1.87 ± 0.04</td>
</tr>
<tr>
<td>N-octyl (compound 2)</td>
<td>48 ± 3</td>
</tr>
</tbody>
</table>

The concentration of CEase was 0.1 mM. Reactions were run at 25.0 ± 0.1°C in 0.1 M sodium phosphate buffer, pH 7.00, that contained 0.1 M NaCl. The concentration of CEase was 0.1 μM. Reactions were run at 25.0 ± 0.1°C in 0.1 M sodium phosphate buffer, pH 7.00, that contained 0.1 M NaCl.
Kinetic runs were done at 25.0 ± 0.1 °C and pH 7.80 in sodium phosphate buffer (0.1 M) that contained NaCl (0.1 M). CEase (14 nM) was incubated with compound 1 or 2 (1 and 0.25 μM, respectively) for 9 min, then diluted 50-fold into buffer or TC/PC micelles (1.87 mM). 20-μl aliquots of the diluted enzyme-inhibitor solution were taken at time intervals and injected into 0.98 ml of buffer that contained 0.2 mM PNPB.

The presence of 0.05 M hydroxylamine increases the first-order rate constant for the return of activity of CEase after inhibition by compound 1 from 5.2 ± 0.2 × 10⁻⁵ s⁻¹ to 2.7 ± 0.3 × 10⁻⁵ s⁻¹, a 5-fold increase (Fig. 3). The apparent first-order rate constant for the return of activity in the absence of hydroxylamine is less than the value given in Table II for the same batch of the porcine enzyme. This is due to the fact that the concentration of compound 1 is 0.67 μM, which inhibits the enzyme with a rate constant of approximately 6 × 10⁻³ s⁻¹, (calculated from the data in Fig. 2). Hydroxylamine has no effect on the spontaneous hydrolysis of compound 1 as the initial rates of the nonezymatic hydrolysis in the presence and absence of 0.05 M hydroxylamine are the same within experimental error (1.8 ± 0.2 × 10⁻³ A s⁻¹ and 1.9 ± 0.1 × 10⁻³ A s⁻¹, respectively, where A = absorbance). Therefore, the increase in the rate constant in the presence of hydroxylamine is due to reactivation of the inhibited enzyme and not to a faster deplation of compound 1.

Cholesteryl-N-alkyl Carbamates—Fig. 4 shows the effect of compounds 3 and 4 on the CEase-catalyzed hydrolysis of PNPB. In the presence of the octyl derivative (compound 4, 3 mol %) the initial rate of the reaction is reduced by 40% after an incubation period of 1 min, and the butyl derivative (compound 3, 4.5 mol %) effected an inhibition of 60% after the same time period of incubation. The inhibition by both compounds is time-dependent but it does not follow simple first-order kinetics. TLC of hexane extracts of micellar solutions of compound 3 or 4, incubated with and without CEase, indicated that these compounds were not hydrolyzed by the enzyme. Interestingly, the commercial bovine pancreatic CEase is not inhibited by cholesteryl-N-octyl carbamate.

Trypsin-catalyzed hydrolysis of PNPB is not affected by the presence of compound 3 or 4, whereas the catalytic activity of chymotrypsin on the same compound is reduced by 20% in the presence of compound 4, but is unaffected by compound 3.

**DISCUSSION**

The results for the p-nitrophenol-N-alkyl carbamates are characteristic of active site-directed irreversible inhibition, since they meet some of the criteria proposed by Abeles and Maycock (19). First, the inhibition is time-dependent and follows first-order kinetics; second, with increasing concentration of inhibitor the porcine enzyme displays saturation kinetics; third, the enzyme can be protected from inhibition by compound 1 and 2 in the presence of a competitive inhibitor. However, our results also show evidence for the hydrolsy of the covalently modified enzyme, which indicates that p-nitrophenyl-N-alkyl carbamates are in fact poor substrates of CEase. We further investigated the CEase-catalyzed hydrolysis of p-nitrophenyl-N-butyl carbamate by looking at the effect of hydroxylamine on k₃, the rate constant for return of activity of inhibited CEase. Significant activation of k₃ by hydroxylamine is observed, which indicates that nucleophilic decarboxamylatation of a rate-limiting N-butyl carbamyl-CEase is occurring. Similar results have been obtained for decarboxamylaion of serine hydrodrolases (20). Hence, the mechanism of inhibition of CEase by carbamates is analogous to the acylzyme mechanism for CEase-catalyzed hydrolysis of esters (8, 9) (see Introduction).

The magnitude of the bimolecular rate constant (k₃/Kₐ) for the inhibition of CEase by compound 1 in homogeneous solution indicates that p-nitrophenyl-N-alkyl carbamates are potent inhibitors. Furthermore, a comparison of the second-
order rate constants for inhibition of the enzyme by compounds 1 and 2 indicates that the octyl derivative is more potent than the butyl. These two compounds also inhibit three serine proteases, chymotrypsin, trypsin, and elastase (14), although compound 2 was found to be a less potent inhibitor than compound 1 for each protease. This indicates that the specificity of inhibitors for CEase, relative to other serine hydrolases present in the pancreas, can be increased by incorporating into the molecule a longer alkyl chain.

A comparison of the rate constant for hydrolysis of the butyl carbamylezyme and $k_{\text{cat}}$ for PNPB (for which deacylation is rate limiting (8)) shows that the carbamylezyme is hydrolyzed 10-fold more slowly than the acylenzyme. Although part of this deceleration may be attributed to the longer chain for the carbamylezyme (6 atoms rather than 4; cf. the difference in the first-order rate constants for the hydrolysis of the N-octyl and N-butyl carbamylezymes given in Table II), most of the effect can be attributed to the increase in the electron density around the carbonyl carbon due to the incorporation of the nitrogen atom. This comparison, therefore, gives a quantitative assessment of the effectiveness of these compounds in inhibiting CEase.

The effect of TC/PC micelles on the interaction of CEase with compounds 1 and 2 is also of some interest. The results in Table II show that the rate constant for the inhibition of CEase by compound 1 is increased in the presence of micelles, but inhibition by compound 2 is decreased. Detergent can affect activity by changing the dispersion of the inhibitor. This has been suggested for bile salt activation of CEase-catalyzed hydrolysis of various substrates (4, 5, 21). The addition of detergents is also known to inhibit the CEase-catalyzed hydrolysis of substrates that are micelle formers, which has been suggested to occur because of surface dilution of the substrate at the interface (5). This may explain the decrease in the rate of the inhibition of CEase by compound 2 in the presence of TC/PC micelles. However, the fact that the inhibition by compound 2 is decreased in the presence of micelles but that by compound 1 is increased can be readily rationalized in terms of the relative lipophilicity of the inhibitors. The more hydrophobic N-octyl carbamate should partition more effectively into the interior of TC/PC micelles, and hence the fraction of compound 2 available for inhibition (i.e. compound 2 in the aqueous phase and at the micelle-water interface) should be less than the corresponding fraction of compound 1. In addition, it appears that the micelles have a direct effect on the enzyme which is dependent on the structure of the substrate. This is signalled by the almost 2-fold increase in the turnover of the butyl carbamylezyme in the presence of micelles. This effect is structure-dependent, since the turnover of the octyl carbamylezyme is not affected by micelles. A more detailed study than that described herein is required to fully characterize the effects of micelles on the kinetics of CEase reactions.

The inhibition of CEase by the cholesteryl-N-alkyl carbamates 3 and 4 is not characteristic of active-site-directed irreversible inhibition, because, although the inhibition shows a slight time dependence, it is not a first-order process. In addition, the enzyme does not hydrolyze the cholesteryl derivatives, which indicates that these carbamates do not interact with the enzyme in the same manner as the p-nitrophenyl derivatives. If CEase is carboxamylated by either carbamate 3 or 4, the resulting carboxamylezymes should be hydrolyzed with the same rate constants as given in Table II, and therefore there should be evidence of turnover. There are two possible explanations for this apparent conflicting information: first, that this is an effect of the way in which the inhibitor is dispersed in solution; second, that the cholesteryl carbamates are unreactive toward nucleophile attack by the proposed serine residue in the active site of the enzyme. It has been suggested that the apparent substrate specificities of lipolytic enzymes reflect the surface activity of the substrate more than the ability of the enzyme to interact with the particular substrate at the interface (5). Therefore, it is possible, knowing the low surface activity of cholesteryl esters (22), that the cholesteryl carbamates are not sufficiently exposed at the interface to allow for the full interaction of the enzyme-active-site with the cholesteryl functionality. However, we think that the second idea is more plausible because cholesteryl is a poor leaving group relative to p-nitrophenol. Also, we find that compound 4 is very stable to base and nucleophiles (no cleavage of the carbamate was detected after 48 h on addition of methanolic KOH to 0.7 M, nor after 6 h at 50 °C with 0.5 M hydroxylamine in aqueous methanol. Only partial cleavage was effected by lithium aluminum hydride in ether at room temperature after 24 h.

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