Kinetic mechanism of *Clostridium perfringens* phospholipase C

Hydrolysis of a thiophosphate analogue of lysophosphatidylcholine

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The hydrolysis of *S*-[2-(hexadecanoyloxy)ethyl]thiophosphocholine (I), an analogue of lysophosphatidylcholine, by *Clostridium perfringens* phospholipase C, was followed at pH 7.5, 37 °C and *I* 1.0 (maintained with KCl), in a continuous assay, by monitoring the reduction of 5,5'-dithiobis-(2-nitrobenzoic acid) at 412 nm. Simple saturation kinetics are observed with linear mixed-type slope-intercept effects for the hydrolysis of compound (I) with variable $[Ca^{2+}]$ at fixed concentrations of compound (I) and a simple slope effect as $[compound (I)]$ is varied at fixed concentrations of Ca^{2+} . These data are consistent with a simple ordered rapid-equilibrium mechanism in which Ca^{2+} binds to the enzyme first followed by substrate. The observed kinetic constants at pH 7.5, 37 °C and *I* 1.0 are $K_1 = 12.0$ mM (Ca^{2+} dissociation), $K_2 = 36$ μ M [compound (I) dissociation] and $V_{max} = 552$ μ M $\cdot$ min⁻¹ $\cdot$ mg⁻¹. Alkane diammonium salts inhibit the enzyme by a non-competitive mechanism that involves binding to free enzyme, $E \cdot Ca^{2+}$ and $E \cdot Ca^{2+} \cdot S$. The use of the simple micellarized substrate under these conditions allows the determination of kinetic and inhibition constants without complications arising from enzyme-micelle interactions.

INTRODUCTION

Phospholipase C (phosphatidylcholine cholinephosphohydrolase, EC 3.1.4.3) catalyses the hydrolysis of the glycerophosphate ester bond in phospholipids, and the enzymes from *Bacillus cereus* and *Clostridium perfringens* have been widely studied and reviewed (Van den Bosch, 1982; Dennis, 1983; Daniel *et al.*, 1988; Jolivet-Reynaud *et al.*, 1988). The gene encoding the enzyme from *C. perfringens* has been cloned and expressed (Okabe *et al.*, 1989; Titball *et al.*, 1989; Tso & Siebel, 1989), and the crystal structure of the enzyme from *B. cereus* has been described (Hough *et al.*, 1989). The activity of the bacterial phospholipase C enzymes has been assayed by many methods (Jolivet-Reynaud *et al.*, 1988), including a continuous spectrophotometric assay utilizing a thiophosphate analogue of dioctanoylglycerophosphocholine, coupled to the reduction of 5,5'-dithiobis-(2-nitrobenzoic acid) (DTNB) (Snyder, 1987*a,b*). This latter method offers a multitude of advantages over previous methods, but simple saturation kinetics are not observed above the substrate critical micelle concentration, making the approach less useful for the determinations of kinetic and inhibition parameters. In order to extend this concept to kinetically simpler substrates, we have examined the kinetics of the hydrolysis of *S*-[2-(hexadecanoyloxy)ethyl]thiophosphocholine (I), described by Cox *et al.* (1979), by *C. perfringens* phospholipase C. We have found this substrate to obey simple Michaelis-Menten kinetics and to be suitable for inhibition studies.

MATERIALS AND METHODS

Materials

All chemicals used were readily available commercial products

and were used without further purification unless otherwise noted; all alkanediamine dihydrochlorides were recrystallized from ethanol before use. *S*-[2-(Hexadecanoyloxy)ethyl]thiophosphocholine (I) was prepared by the method described by Cox *et al.* (1979), as modified by Snyder (1987*c*). *C. perfringens* phospholipase C (300 units/mg of protein) was from Sigma Chemical Co. and was used as supplied.

Kinetics

The critical micelle concentration of compound (I) was determined by the binding of Rhodamine 6G (Steinhardt *et al.*, 1974). At 37 °C and an ionic strength of 0.15 the critical micelle concentration for compound (I) was estimated to be 20 μ M, decreasing to 10 μ M at an ionic strength of 1.0 (maintained with KCl). Kinetic determinations were performed at 37 °C, *I* 1.0 (maintained with KCl), $[S] > 20$ μ M and pH 7.5 (10 mM-Mops) with the use of a Hitachi 100-40 spectrophotometer equipped with an automatic cell changer and a thermostatically controlled cell compartment, by monitoring the reduction of DTNB at 412 nm. All kinetic determinations were run in duplicate and kinetic parameters were determined by non-linear least-squares fit of the data to the appropriate equation. No induction period was observed during initial-rate studies. The initial rate of hydrolysis of compound (I) was found to be a simple linear function of enzyme concentration over the concentration range $[E] = 12$ –96 μ g/ml, based on enzyme solid, as supplied.

RESULTS AND DISCUSSION

The critical micelle concentration of the substrate compound (I) was estimated by Rhodamine 6G binding (Steinhardt *et al.*, 1974) to be 20 μ M at 37 °C at an ionic strength of 0.15, decreasing to 10 μ M at an ionic strength of 1.0 (maintained with KCl). Kinetic determinations in this study were performed at *I* 1.0 and $[S] > 20$ μ M to minimize enzyme-monomer interactions. The hydrolysis of the thioester linkage in compound (I) by *C. perfringens* phospholipase C results in the release of phosphocholine and 1 equivalent of free thiol (Scheme 1) (Cox *et*

Abbreviations used: DTNB, 5,5'-dithiobis-(2-nitrobenzoic acid); TNB⁻, 5-mercapto-2-nitrobenzoate anion.

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al., 1979, Riedy & Snyder, 1987). In the presence of DTNB the thiol produced reacts to release 1 molar equivalent of 5-mercapto-2-nitrobenzoate (TNB⁻ anion) and forms a mixed disulphide. This intermediate can react with a second molar equivalent of free thiol, releasing TNB⁻ anion and forming the disulphide shown in Scheme 1. The initial velocity of DTNB reduction, monitored at 412 nm in the presence of 70 μM -compound (I) (37 °C, I 1.0 and $[\text{Ca}^{2+}] = 50 \text{ mM}$) was found to follow simple saturation kinetics with increasing DTNB concentration, with an apparent half-maximum at $[\text{DTNB}] = 20 \mu\text{M}$; kinetic determinations were routinely performed at $[\text{DTNB}] = 500 \mu\text{M}$, where the reaction rate is independent of $[\text{DTNB}]$. Under the conditions stated above (37 °C, I 1.0, $[\text{Ca}^{2+}] = 50 \text{ mM}$ and $[\text{DTNB}] = 500 \mu\text{M}$), the rate of hydrolysis of compound (I) by *C. perfringens* phospholipase C increases as a simple linear function of ionic strength over the range I 0.15–1.5 according to the equation:

$$v = I(5.9 \mu\text{M}/\text{min}) + 10.5 \mu\text{M}/\text{min}$$

A linear dependence on enzyme concentration (based on enzyme solid) was also observed over the concentration range $[E] = 12\text{--}96 \mu\text{g}/\text{ml}$, extrapolating through the origin.

Unlike data that are commonly observed for enzymes functioning at a micellar interface, initial velocities for the enzyme-catalysed hydrolysis of compound (I) follow simple saturation kinetics under the standard conditions cited above, and generate linear reciprocal plots, as shown in Fig. 1. Linear mixed-type slope-intercept effects are observed for the hydrolysis of compound (I) with variable $[\text{Ca}^{2+}]$ at fixed concentration of compound (I), and a simple slope effect is observed as $[\text{compound (I)}]$ is varied at several fixed concentrations of Ca^{2+} . These data are consistent with a simple ordered rapid-equilibrium mechanism in which Ca^{2+} binds to the enzyme first followed by substrate (Segel, 1975), and suggest that interfacial adsorption of the enzyme is fast relative to substrate binding and turnover. The model and the corresponding velocity equation (1) are shown below:

Plots of $1/v_0$ against $1/[\text{S}]$ for different values of $[\text{Ca}^{2+}]$ have x -intercepts of approx. $1/\{K_2(1 + K_1/[\text{Ca}^{2+}])\}$, so that a plot of the reciprocals of these intercepts against $1/[\text{Ca}^{2+}]$ allows K_1 and K_2 ,

Fig. 1. Effect of substrate concentration on the hydrolysis of compound (I) catalysed by *C. perfringens* phospholipase C

(a) The continuous line is drawn for the theoretical approach to $V_{\max}$ with a K_m of $36 \mu\text{M}$. Assays were under the following conditions: pH 7.5, 37 °C, I 1.0 (maintained with KCl), $[\text{Ca}^{2+}] = 50 \text{ mM}$ and $[\text{DTNB}] = 500 \mu\text{M}$, and the reduction of DTNB was monitored at 412 nm. (b) Double-reciprocal plot of the data from (a).

the dissociation constants for Ca^{2+} and substrate respectively, to be determined. For the hydrolysis of compound (I) these values are $K_1 = 12.0 \text{ mM}$, $K_2 = 36 \mu\text{M}$ and $V_{\max} = 552 \mu\text{M} \cdot \text{min}^{-1} \cdot \text{mg}^{-1}$.

The fact that simple saturation kinetics are observed with the thiophosphate substrate suggests that this system may be useful for the determination of kinetic inhibition constants for this enzyme. It has been reported that both phosphatidylinositol-

Scheme 1. Hydrolysis of *S*-[2-(hexadecanoyloxy)ethyl]thiophosphocholine by phospholipase C

specific and broad-specificity phospholipases C are inhibited by cations including polyamines such as spermine, spermidine and putrescine (Sechi *et al.*, 1978; Sagawa *et al.*, 1983; Wojcikiewicz & Fain, 1989; Yeats & Bakhle, 1990). Preliminary work with the *C. perfringens* enzyme demonstrated similar (but weak) inhibition by putrescine, but striking inhibition by 1,12-diaminododecane. In order to quantify this further, we have examined inhibition by a series of alkane diamines with 6, 8, 10 and 12 intervening carbon atoms. For all compounds examined, mixed slope-intercept effects were observed in double-reciprocal plots with concentrations of either substrate or Ca^{2+} varied at fixed concentrations of inhibitors (e.g. Fig. 2). These data are most consistent with the model and rate equation (2) (Segel, 1975) shown below in which the inhibitor binds to all enzyme forms including the $\text{E} \cdot \text{Ca}^{2+} \cdot \text{S}$ complex:

Inspection of eqn. (2) shows that the equilibrium constant for the binding of inhibitor to the ternary complex, $\alpha \beta K_1$, is readily obtained from replots of the y -intercept of double-reciprocal plots of velocity versus [compound (I)] and that the constant for EI dissociation, K_1 , is obtained from replots of slopes of reciprocal plots of velocity versus $[\text{Ca}^{2+}]$. Values of $\alpha \beta K_1$ for all four amine inhibitors and values of K_1 , α and β for the C_8 and C_{12} compounds are collected in Table 1. The relationship between the dissociation constant for the inhibitor-ternary complex, $\alpha \beta K_1$, and the length of the intervening alkane chain is most clearly demonstrated by the plot in Fig. 3, in which $\ln(\alpha \beta K_1)$ is plotted against the alkane chain length. In a plot such as this, the observed slope is a function of the utilization of hydrophobic binding energy towards the formation of the inhibitory complex. (Gitler & Ochoa-Solano, 1968; Young & Hou, 1979; Young & Briedis, 1990). For the data in Fig. 3 the slope is $\Delta G \approx -2.01$ kJ/mol of methylene unit (-480 cal/mol of methylene unit). This value compares with the value of $\Delta G \approx -2.72$ kJ/mol of methylene unit (-650 cal/mol of methylene unit), which has been estimated for the free-energy change resulting from the transfer of a methylene unit from free solution to the micellar phase (Gitler & Ochoa-Solano, 1968) and suggests that, in the present case, approx. $2.01/2.72 = 74\%$ of the available hydrophobic binding energy has been utilized for the formation of the inhibitor complex. The isolated constants α and β for the C_8 and C_{12} compounds further suggest that the increase in chain length is reducing the ability of the enzyme to bind Ca^{2+} (α decreases from 0.87 to 0.23) while β is essentially constant and large. The magnitude of β means that, once the $\text{E} \cdot \text{Ca}^{2+} \cdot \text{I}$ complex forms, binding of substrate is strongly inhibited. The inhibition is, however, not competitive with respect to substrate, perhaps suggesting a conformational change or binding of the inhibitor at a site that interferes with $\text{E} \cdot \text{Ca}^{2+}$ micelle interactions.

A number of methods have been utilized to assay the activity of *C. perfringens* phospholipase C and to evaluate inhibitors. These include a haemolytic assay, measuring the release of haemoglobin from rabbit erythrocytes, a turbidimetric method using egg phosphatidylcholine/sodium cholate dispersions,

Fig. 2. Double-reciprocal plots of $1/v$ versus $1/[S]$ for the hydrolysis of compound (I) catalysed by *C. perfringens* phospholipase C at the following fixed concentrations of 1,8-diamino-octane: □, 0 mM; ■, 10 mM; ●, 20 mM; ○, 36 mM.

Assays were under the following conditions: pH 7.5, 37 °C, I 1.0 (maintained with KCl), $[\text{Ca}^{2+}] = 50$ mM and $[\text{DTNB}] = 500$ μM, and the reduction of DTNB was monitored at 412 nm. Inset: Replot of y -intercept versus $[1,8\text{-diamino-octane}]$.

titrimetric methods and most commonly monitoring the hydrolysis of *p*-nitrophenylphosphocholine (Jolivet-Reynaud *et al.*, 1988). This compound, although widely used, is a poor substrate for the enzyme, has a large K_m and a low $V_{\max}$ (approx. 42 mM and 59 μM·min⁻¹·mg⁻¹ respectively) and, since it exists as a monomer in solution, would not provide kinetic information regarding $\text{E} \cdot \text{Ca}^{2+}$ micelle interactions. Snyder (1987a,b) has described the use of the thiophosphate analogue of dioctanoyl-glycerophosphocholine in a continuous spectrophotometric assay for the *B. cereus* phospholipase C. This substrate has a higher critical micelle concentration than compound (I) (56 μM compared with 10 μM) and the *R*- and *S*-enantiomers undergo hydrolysis at significantly different rates, producing complex time-dependencies and non-Michaelis-Menten behaviour (Riedy

Table 1. Inhibition by diaminoalkanes of the hydrolysis of compound (I) catalysed by *C. perfringens* phospholipase C

Assays were under the following conditions: pH 7.5, 37 °C, I 1.0 (maintained with KCl), $[\text{Ca}^{2+}] = 50$ mM and $[\text{DTNB}] = 500$ μM, and the reduction of DTNB was monitored at 412 nm. $\alpha \beta K_1$ is the equilibrium constant for the dissociation of inhibitor from the $\text{E} \cdot \text{Ca}^{2+} \cdot \text{S} \cdot \text{I}$ complex. K_1 is the dissociation constant for the $\text{E} \cdot \text{I}$ complex. α is the dissociation constant of inhibitor from $\text{E} \cdot \text{Ca}^{2+} \cdot \text{I}$ complex, as a fraction of that from the free enzyme; β is the factor by which the dissociation constant of inhibitor from the $\text{E} \cdot \text{Ca}^{2+} \cdot \text{I}$ complex is increased by the binding of substrate.

Inhibitor	$\alpha \beta K_1$ (mM)	K_1 (mM)	α	β
$^+\text{H}_3\text{N}-(\text{CH}_2)_6-\text{NH}_3^+$	1440	31	0.87	25
$^+\text{H}_3\text{N}-(\text{CH}_2)_8-\text{NH}_3^+$	680	—	—	—
$^+\text{H}_3\text{N}-(\text{CH}_2)_{10}-\text{NH}_3^+$	138	5.6	0.23	23
$^+\text{H}_3\text{N}-(\text{CH}_2)_{12}-\text{NH}_3^+$	30	—	—	—

Fig. 3. Plot depicting the dependence on number of methylene units of the inhibition constant $\alpha\beta K_i$ for diamine inhibition of the hydrolysis of compound (I) catalysed by *C. perfringens* phospholipase C

Assays were under the following conditions: pH 7.5, 37 °C, I 1.0 (maintained with KCl), $[Ca^{2+}] = 50$ mM and $[DTNB] = 500$ μ M, and the reduction of DTNB was monitored at 412 nm. The slope gives a value of $\Delta G^0 = -2.01$ kJ/mol of methylene unit (-480 cal/mol of methylene unit).

& Snyder, 1987). The fact that the substrate used in the present case follows simple saturation kinetics at substrate concentrations above the critical micelle concentration probably means that the formation and dissociation of the enzyme·micelle· Ca^{2+} complex is fast relative to substrate binding and turnover. The relatively large dissociation constant for Ca^{2+} suggests that the bivalent metal ion may be binding the micellar substrate to form a relatively weak micelle· Ca^{2+} pre-association complex, which then binds enzyme. The low Michaelis constant for compound (I), relatively high turnover number and simple kinetic presentation make this substrate uniquely suitable for inhibition and activation studies.

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