Theoretical approach to the steady-state kinetics of a bi-substrate acyl-transfer enzyme reaction that follows a hydrolysable-acyl-enzyme-based mechanism

Application to the study of lysophosphatidylcholine:lysophosphatidylcholine acyltransferase from rabbit lung

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A kinetic model is proposed for catalysis by an enzyme that has several special characteristics: (i) it catalyses an acyl-transfer bi-substrate reaction between two identical molecules of substrate, (ii) the substrate is an amphiphilic molecule that can be present in two physical forms, namely monomers and micelles, and (iii) the reaction progresses through an acyl-enzyme-based mechanism and the covalent intermediate can react also with water to yield a secondary hydrolytic reaction. The theoretical kinetic equations for both reactions were deduced according to steady-state assumptions and the theoretical plots were predicted. The experimental kinetics of lysophosphatidylcholine:lysophosphatidylcholine acyltransferase from rabbit lung fitted the proposed equations with great accuracy. Also, kinetics of inhibition by products behaved as expected. It was concluded that the competition between two nucleophiles for the covalent acyl-enzyme intermediate, and not a different enzyme action depending on the physical state of the substrate, is responsible for the differences in kinetic pattern for the two activities of the enzyme. This conclusion, together with the fact that the kinetic equation for the transacylation is quadratic, generates a 'hysteretic' pattern that can provide the basis of self-regulatory properties for enzymes to which this model could be applied.

INTRODUCTION

The kinetic behaviour of enzymes that catalyse bi-substrate reactions has been exhaustively discussed (Cornish-Bowden, 1976; Cleland, 1977, 1979; Segel, 1979), including the patterns of their inhibition by substrates and products. However, there exist enzymes where the concurrence of several special factors leads to kinetic patterns that have not been analysed in the literature. Although the theoretical treatment of these special cases may seem wasteful, the growing number of complex enzymes actually known could make the kinetic analysis of such special cases important.

Lysophosphatidylcholine:lysophosphatidylcholine acyltransferase is the enzyme responsible for the transacylation involved in the mobilization of phosphatidylcholines from pulmonary surfactant. This enzyme, present in the soluble fraction of lung tissue, catalyses acyl transfer between two lysophosphatidylcholine molecules to yield phosphatidylcholine and glycerophosphorylcholine (Oldenburg & van Golde, 1976). Previous studies have reported data on the kinetics and mechanism of action of lysophosphatidylcholine:lysophosphatidylcholine acyltransferase from rabbit lung (Casals *et al.*, 1982, 1984a,b; Arche *et al.*, 1984; Pérez-Gil *et al.*, 1989a,b).

According to the classification proposed by Cleland

(1963), the reaction catalysed by lysophosphatidylcholine:lysophosphatidylcholine acyltransferase is a Bi Bi reaction with identical substrate molecules. The enzyme catalyses concurrently the hydrolysis of lysophosphatidylcholine and the utilization of the released fatty acid for phosphatidylcholine synthesis (Abe *et al.*, 1974; Brumley & van den Bosch, 1977; Casals *et al.*, 1982). An acyl-enzyme intermediate has been proposed for the reaction (Vianen & van den Bosch, 1978; Van Heusden & van den Bosch, 1979), and in this case the reaction should follow a Ping Pong kinetic mechanism.

It is well known that the substrate of this enzyme, lysophosphatidylcholine, is an amphiphilic molecule that can adopt different physical structures in aqueous solutions. Above the critical micellar concentration (CMC) the main structure is the micelle, but, in spite of its lytic properties against lipid bilayers, lysophosphatidylcholine can be embedded in phospholipid membranes. The importance of the physical state of the substrate on the transacylation and hydrolytic activities of the enzyme has been suggested previously (Brumley & van den Bosch, 1977; Van Heusden *et al.*, 1980). Then it was proposed that the hydrolytic reaction would be a consequence of reaction with the monomeric form of substrate whereas transacylation would come from reaction with micelles (Casals *et al.*, 1982), and indeed the concentration of micelles was found to modify the

Abbreviation used: CMC, critical micellar concentration.

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hydrolysis/transacylation ratio (Casals *et al.*, 1984a). According to this hypothesis, the initial-velocity plots for hydrolysis and transacylation fitted to a hyperbola and a sigmoid curve respectively. The inflexion point of the sigmoid curve should be coincident with the CMC of lysophosphatidylcholine under the experimental conditions and therefore it was possible to obtain the kinetic parameters K_m and V for both reactions (Casals *et al.*, 1982).

In the present investigation a detailed steady-state kinetic study was carried out with enzyme preparations of high specific activity that were obtained by an improved isolation procedure; also, the time of reaction was decreased with respect to the assay previously described (Casals *et al.*, 1982). The kinetics of hydrolysis and transacylation rates do not accord with the previous hypothesis.

As described above, lysophosphatidylcholine: lysophosphatidylcholine acyltransferase seems to proceed by a Ping Pong Bi Bi kinetic mechanism. On this basis we propose a kinetic model in which hydrolysis and transacylation activities are the result of nucleophilic competition between water and lysophosphatidylcholine

Scheme 1.

and the derived kinetic equations are:

$$v_{HD}/[E]_0 = (a + b[S])k_{+3}/([S]^2 + c[S] + d) \quad (1)$$

$$v_{AT}/[E]_0 = (a + b[S])(e + f[S])/([S]^2 + c[S] + d) \quad (2)$$

where v_{HD} and v_{AT} are the initial rates of hydrolysis and acyl-transfer reaction respectively, $[E]_0$ is the initial concentration of enzyme and the coefficients a – f have the following values:

$$\begin{aligned} a &= K_{m1}K_{m2}k_{+2}[S_m]/[K_{m1}'(k_{+2} + k_{+5})] \\ b &= K_{m2}k_{+2}/(k_{+2} + k_{+5}) \\ c &= k_{+5}K_{m1} + K_{m2}(k_{+2} + k_{+3}) + [S_m](k_{+2}K_{m2}/K_{m2}' + k_{+2}'K_{m1}/K_{m2} + k_{+5}K_{m1}/K_{m1}' + k_{+5}'K_{m2}/K_{m2}') \\ d &= K_{m1}K_{m2}\{k_{+3} + [S_m]((k_{+2} + k_{+3})/K_{m1}' + k_{+5}'/K_{m2}') + (k_{+2}' + k_{+5}')/K_{m1}'K_{m2}'\} \\ e &= k_{+5}'[S_m]/K_{m2}' \\ f &= k_{+5}/K_{m2} \end{aligned}$$

for the acyl-enzyme. As the water concentration is constant, the hydrolysis mechanism may be described as a Crypto Ping Pong Uni Bi mechanism (Roberts, 1977). Since aqueous solutions of pure lysophosphatidylcholine are used as substrate, competition between monomers and micelles is also considered.

The aim of the present work is to deduce the theoretical steady-state equations for this model and to check its validity when applied to the study of lysophosphatidylcholine:lysophosphatidylcholine acyltransferase from rabbit lung. The validation of the model should be useful for the study of other similar enzymes.

THEORY

The model of the reaction is based on a Ping Pong Bi Bi mechanism with two identical substrate molecules and with the acyl-enzyme yielding two different products as a result of competition between two nucleophiles in the deacylation step of the mechanism. Also, the substrate can be in two different physical states, monomers and micelles. This kinetic mechanism is depicted in Scheme 1, where E is the free enzyme and F the acyl-enzyme, S_m is the monomeric form of the substrate, S is the micellar form of the substrate, P is the first product and Q and R are the products of the hydrolysis and the acyl-transfer reactions respectively. It is assumed that catalytic steps are irreversible. It should also be noted that k_{+3} is an apparent constant that includes the concentration of water.

According to steady-state assumptions:

$$d[E \cdot S_m]/dt = d[E \cdot S]/dt = d[F]/dt = d[F \cdot S_m]/dt = d[F \cdot S]/dt = 0$$

where K_{m1} and K_{m2} are the Michaelis constants for the first and second micellar substrate molecules respectively and K_{m1}' and K_{m2}' are the same constants when the substrate is monomeric.

The considerable complexity of these equations impedes their direct application to the experimental data. Therefore some simplification is necessary, and one possibility to be considered is that substrate is only in micellar form. This assumption is essentially true for substrate concentration ranges much higher than the CMC. (Analysis of the situation where the substrate is considered to be solely in the monomeric state is considered separately below.) Thus, if $[S_m] = 0$, the kinetic equations can be simplified to:

$$v_{HD} = V_{HD}[S]/D \quad (3)$$

$$v_{AT} = V_{AT}[S]^2/D \quad (4)$$

where V_{HD} and V_{AT} are kinetic constants for hydrolysis and transacylation respectively, related to maximum velocity, and have the values:

$$V_{HD} = k_{+2}k_{+3}K_{m2}[E]_0/(k_{+2} + k_{+5})$$

and

$$V_{TA} = k_{+2}k_{+5}[E]_0/(k_{+2} + k_{+5})$$

and the denominator is:

$$D = [S]^2 + B[S] + A$$

Enzyme assay

Several modifications were introduced into the activity assay method previously described (Casals *et al.*, 1982). Both enzyme activities, transacylation and hydrolysis, were assayed simultaneously in the same incubation mixture containing the substrate, 1-[1-¹⁴C]palmitoyl-*sn*-glycero-3-phosphocholine (sp. radioactivity 0.05 Ci/mol), sonicated for 30 s at 12 μ M in an MSE sonicator in 0.1 ml of 0.1 M-potassium phosphate buffer, pH 6.8, and 0.1 ml of enzyme solution. In the experiments on inhibition by products, the appropriate amount of product was included, before sonication, in the same total volume of buffer. The mixture was incubated at 37 °C for 1 min because it was found that with incubation times longer than 1 min the initial rates for both activities of the

procedure, in which 2-mercaptoethanol is carboxy-methylated to prevent absorption at the wavelength of measurement. Horse serum albumin was used as standard.

RESULTS

Initial rates of the hydrolysis and transacylation activities of lysophosphatidylcholine:lysophosphatidylcholine acyltransferase are plotted versus substrate concentration in Fig. 2. The shapes of the curves agree with those presented in Fig. 1: sigmoidal behaviour for acyl transfer and a curve with a maximum for hydrolysis. The data were fitted to the theoretical eqns. (3) and (4) and experimental parameters were calculated, giving the following values:

$$\begin{aligned} A &= (1.2 \pm 0.1) \times 10^4 \mu\text{M}^2 \\ B &= 25 \pm 10 \mu\text{M} \\ V_{\text{HD}} &= (3.5 \pm 0.08) \times 10^4 \mu\text{M} \cdot (\text{nmol/min per mg of protein}) \\ V_{\text{AT}} &= 75.0 \pm 5.0 \text{ nmol/min per mg of protein} \end{aligned}$$

enzyme were underestimated. The standard concentration of enzyme solution used in all the experiments was 0.5 mg/ml.

Determination of the CMC of lysophosphatidylcholine

The CMC of lysophosphatidylcholine, either alone or in the presence of enzyme, was determined according to the method of Barden & Cleland (1969), by measuring the increase of absorbance at 610 nm caused by the adsorption of pinacyanol chloride on the micelles of the lysophospholipid.

Kinetic data

All kinetic measurements were made in triplicate incubations. The experimental results were fitted to the corresponding kinetic equations by the non-linear-regression facility of the statistical software package BMDP (Dixon, 1981). The resultant parameters are expressed with their standard errors.

Protein determination

Protein concentration was determined by the Ross & Schatz (1973) modification of the general Lowry

As predicted from the theoretical equations, the transacylation/hydrolysis ratio was linearly dependent on the substrate concentration (Fig. 2b).

These results point to the significance of the proposed model in explaining the dual kinetic behaviour of the enzyme, at least when the substrate concentration is above its CMC. Moreover, they demonstrate that the physical state of substrate is not the cause of the differences in kinetic behaviour between the two activities of lysophosphatidylcholine:lysophosphatidylcholine acyltransferase.

To confirm that the kinetic differences were not due to the physical state of substrate, the kinetic analysis was carried out at substrate concentrations around the CMC. There is no agreement in the literature about the CMC of lysophosphatidylcholine in aqueous solutions (Hamori & Michaels, 1971; Wassef *et al.*, 1978; Weltzien, 1979). We calculated this parameter in the conditions of our experiment and obtained the value of 7 μ M, either in the presence or in the absence of enzyme. This value agrees with that proposed by Wassef *et al.* (1978) and Haberland & Reynolds (1975).

Lysophosphatidylcholine:lysophosphatidylcholine

Fig. 2. (a) Experimental kinetics of hydrolysis (○) and transacylation (●) activities of lysophosphatidylcholine:lysophosphatidylcholine acyltransferase from rabbit lung versus substrate concentration and (b) linear dependence of transacylation/hydrolysis ratio versus substrate concentration

Fig. 3. Kinetics of hydrolysis (○) and transacylation (●) in the micromolar range of substrate concentration

Broken lines represent the asymptotic slopes for hydrolysis rate above and below the CMC; the intercept of both slopes corresponds to the breakpoint of the curve and its coincident with CMC of lysophosphatidylcholine.

acyltransferase kinetics in the micromolar range of lysophosphatidylcholine concentration are shown in Fig. 3. It is worth noting that both activities, hydrolysis and transacylation, are present when the concentration of substrate is below the CMC, indicating that the enzyme also catalyses transacylation reaction with monomers of lysophosphatidylcholine. Moreover, hydrolysis activity does not remain constant above CMC, as it would if this reaction were dependent on substrate monomers. These results suggest that the enzyme can hydrolyse lysophosphatidylcholine in the micellar state. Furthermore, the fact that the curves plotted in Fig. 3 had a greater slope above than below the CMC of substrate is a consequence of the different kinetic parameters for monomeric and micellar substrate. Similar results for the acyl-transfer reaction were not obtained because of the very low transacylation activity.

The proposed model can be more extensively assessed by studying the patterns of inhibition by products. According to the mechanism depicted in Scheme 2, it could be expected that the final products from both reactions, free fatty acid and phosphatidylcholine, behave as competitive inhibitors because they are in equilibrium with free enzyme. On the other hand, the product obtained in the common step, i.e. glycerophosphorylcholine, should be in equilibrium with acyl-enzyme, giving a non-competitive inhibition pattern. From eqns. (5) and (7), which describe the apparent kinetic parameters in the presence of inhibitor, it is concluded that the variation of the A/V_{HD} ratio versus inhibitor concentration allows us to determine the existence of a competitive pattern and the calculation of the correspondent K_{I1} . The possible sequestration of the intermediate F by the first product or an analogue could be deduced and the subsequent inhibition constant K_{I2} calculated from the variation of kinetic parameter A versus inhibitor concentration.

A/V_{HD} ratio is plotted versus the inhibitor concentration in Fig. 4 for the three products assayed, glycerophosphorylcholine, dipalmitoylphosphatidylcholine and palmitate. This plot is similar to the Dixon (1953)

Fig. 4. Plots of parameter A/V_{HD} versus inhibitor concentration for (a) dipalmitoyl phosphatidylcholine (DPPC), (b) palmitate and (c) glycerophosphorylcholine

plot, where K_i is obtained from the intercept with abscissa axis. As expected, the final products of hydrolysis and transacylation activities exhibited a typical competitive pattern where $K_{I1} = 3.37 \pm 0.39$ mM for inhibition by palmitic acid and $K_{I1} = 0.55 \pm 0.15$ mM for inhibition by dipalmitoylphosphatidylcholine.

By contrast, the presence of glycerophosphorylcholine did not change the A/V_{HD} ratio or any other kinetic parameters of the enzyme at the assayed concentrations.

DISCUSSION

Apart from proteinases, little attention has been paid to analysis of the kinetics and mechanism of acyl-transfer reactions. In this context, lysophosphatidylcholine:lysophosphatidylcholine acyltransferase is a pertinent model because it is an acyl-transfer enzyme and shows special kinetic features as a consequence of the amphiphilic nature of its substrate.

Concerning this enzyme, many groups have focused their attention on the physiological role of lysophosphatidylcholine:lysophosphatidylcholine acyltransferase in the metabolism of pulmonary surfactant (Abe *et al.*, 1974; Tsao & Zachman, 1977; Batenburg *et al.*, 1979; Crecelius & Longmore, 1984; de Vries *et al.*, 1985). However, when the first characterization of the enzyme was reported (Brumley & van den Bosch, 1977; Tsao & Zachman, 1977; van den Bosch *et al.*, 1981), only

preliminary data about the kinetic mechanism were analysed.

Kinetic studies on lysophosphatidylcholine:lysophosphatidylcholine acyltransferase proved to be critically dependent on two questions: (i) the utilization of really pure and native fractions of enzyme and (ii) the selection of adequate conditions for assay of enzymic activity in order to obtain true initial velocities for both activities. The improvement of the isolation and assay methods allowed us to obtain truly reliable data whose interpretation fits the model presented in this paper. It is necessary to point out that the present results are not in agreement with previous reported data on this enzyme. On the basis of all the evidence described in the Results section, one can discard the possibility of exclusive dependence of both activities of the enzyme on the physical form of substrate, as our group previously proposed (Casals *et al.*, 1982, 1984a).

The mechanism proposed in the present paper is able to explain completely the different behaviour of hydrolysis and transacylation experimentally determined. From eqns. (3) and (4), if the enzyme did not bind the second molecule of substrate ($K_{m2} = \infty$), transacylation would vanish and hydrolysis kinetics would show hyperbolic behaviour. Alternatively, if the acyl-enzyme would not be hydrolysed, ($k_{+3} = 0$), transacylation kinetics would also show hyperbolic behaviour. Therefore the idea is supported that the special dual behaviour of hydrolysis and transacylation activities is essentially due to competition between water and substrate for the nucleophilic attack on the intermediate acyl-enzyme and not to the physical state of the substrate.

With respect to the experiments on product inhibition of lysophosphatidylcholine:lysophosphatidylcholine acyltransferase, it is really significant that the final products behave as predicted. The non-inhibiting effect of glycerophosphorylcholine (Fig. 4c) can be explained to be a consequence of the direct participation of water in the reaction (Roberts, 1977). The binary complex acyl-enzyme-glycerophosphorylcholine would in effect never exist because the acyl-enzyme is hydrolysed very fast, owing to the high concentration of water always present, or, when its concentration is high enough, is bound to a second molecule of lysophosphatidylcholine, giving the transacylation reaction.

Several considerations can be made as a result of all the evidence that corroborates the model of kinetic mechanism proposed for lysophosphatidylcholine:lysophosphatidylcholine acyltransferase. Although this mechanism seems to be similar to previously published models (Wykle *et al.*, 1980; Casals *et al.*, 1982), it essentially differs on the origin of the differential behaviour for hydrolysis and transacylation. As mentioned above, the different kinetic patterns of both activities of the enzyme can be attributed not to the polymorphism of substrate but to the competition between water and the second molecule of substrate for the common acyl-enzyme intermediate. Thus both activities cannot be studied independently of each other, as would be possible if the two reactions were governed by different kinetic parameters. Both reactions of this enzyme should be studied as a single catalytic event with unified kinetic parameters.

The physical state of the substrate could play an important role if we focus on the interactions involved in the formation of the enzyme-substrate complex. In fact,

the catalytic parameters seem to be different for both monomers and micelles. At all events, the concentration of each physical form of lysophosphatidylcholine and its participation in the reaction would be dependent on the physiological concentrations of lysophospholipid that lysophosphatidylcholine:lysophosphatidylcholine acyltransferase finds in type II cells.

In view of the membranolytic properties of lysophosphatidylcholine (Weltzien, 1979), the existence of very high concentrations of this lysophospholipid in the pneumocyte is very unlikely. Taking into account the kinetic behaviour of lysophosphatidylcholine:lysophosphatidylcholine acyltransferase and the low concentrations of substrate, it is suggested that its role would be essentially hydrolytic, as protection against the lytic ability of lysophosphatidylcholine. However, if high concentrations of lysophospholipid were present, e.g. in the surfactant-recycling process (Hallman *et al.*, 1981; Jacobs *et al.*, 1983), the protective role of lysophosphatidylcholine:lysophosphatidylcholine acyltransferase could be accompanied by an additional and energetically inexpensive source of surfactant disaturated phosphatidylcholine. This would provide an interesting case of self-regulatory functionality of an enzyme depending on the concentration of substrate, which essentially determines the hydrolysis/transacylation ratio. At all events, whatever the significance this mechanism has in the regulation of the biosynthesis of phosphatidylcholine in lung surfactant, lysophosphatidylcholine:lysophosphatidylcholine acyltransferase can certainly be defined as an hysteretic enzyme, that is it responds slowly, in terms of kinetic behaviour, to a rapid change in substrate concentration (Frieden, 1970). Several mechanisms other than binding have been proposed in order to explain the allosteric behaviour of important enzymes in metabolic regulation, and it has been suggested that in many cases allostery may simply be a kinetic rather than a binding phenomenon (Volkenstein & Goldstein, 1966; Sweeny & Fisher, 1968). In this sense, lysophosphatidylcholine:lysophosphatidylcholine acyltransferase would represent a new case of hysteretic enzyme on the basis of steady-state kinetics derived from its special mechanism. The present work supplies the theoretical basis for the analysis of similar enzymes.

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