Chemical mechanism of lysophosphatidylcholine:lysophosphatidylcholine acyltransferase from rabbit lung

pH-dependence of kinetic parameters

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Lysophosphatidylcholine:lysophosphatidylcholine acyltransferase is an enzyme that catalyses two reactions: hydrolysis of lysophosphatidylcholine and transacylation between two molecules of lysophosphatidylcholine to give disaturated phosphatidylcholine. Following the kinetic model previously proposed for this enzyme [Martín, Pérez-Gil, Acebal & Arche (1990) *Biochem. J.* **266**, 47–53], the values of essential pK values in free enzyme and substrate–enzyme complexes have now been determined. The chemical mechanism of catalysis was dependent on the deprotonation of a histidine residue with pK about 5.7. This result was supported by the perturbation of pK values by addition of organic solvent. Very high and exothermic enthalpy of ionization was measured, indicating that a conformational re-arrangement in the enzyme accompanies the ionization of the essential histidine residue. These results, as well as the results from previous studies, enabled the proposal of a chemical mechanism for the enzymic reactions catalysed by lysophosphatidylcholine:lysophosphatidylcholine acyltransferase from rabbit lung.

INTRODUCTION

Lysophosphatidylcholine:lysophosphatidylcholine acyltransferase is an important enzyme that has been related with the remodelling process of pulmonary surfactant phosphatidylcholine (Abe *et al.*, 1974; Oldenburg & van Golde, 1976). It exhibits two simultaneous activities: (i) transacylation between two molecules of lysophosphatidylcholine to give phosphatidylcholine and glycerophosphocholine and (ii) hydrolysis of lysophosphatidylcholine to yield free fatty acid and glycerophosphocholine. Although several authors have reported studies on the role of this enzyme on the physiology of the lung (Tsao & Zachman, 1977; Crecelius & Longmore, 1984; de Vries *et al.*, 1985), little attention has been paid to its chemical and catalytic mechanism. The kinetic approach could supply relevant information towards an understanding of the real significance of lysophosphatidylcholine:lysophosphatidylcholine acyltransferase in the metabolism of lung surfactant.

Several catalytic properties of lysophosphatidylcholine:lysophosphatidylcholine acyltransferase from rat lung have been reported and an acyl-enzyme-based mechanism has been proposed (Brumley & van den Bosch, 1977; van Heusden & van den Bosch, 1979; van den Bosch *et al.*, 1981). We have previously studied some catalytic properties of lysophosphatidylcholine:lysophosphatidylcholine acyltransferase from rabbit lung, mainly focusing our attention on structural data (Casals *et al.*, 1982, 1984a; Arche *et al.*, 1984), substrate specificity (Casals *et al.*, 1984b) and essential residues (Pérez-Gil *et al.*, 1989a,b, 1990). Also, we have reported a kinetic mechanism for lysophosphatidylcholine:lysophosphatidylcholine acyltransferase from rabbit lung (Martín *et al.*, 1990). It was concluded that the competition between two nucleophiles for the covalent acyl-enzyme intermediate, and not the physical state of the substrate, is responsible for the differences observed in the kinetic patterns of both activities of the enzyme.

The dependence on pH of steady-state kinetic parameters provides information about the ionizable groups involved either in substrate-binding or in catalysis (Cleland, 1977). Both ac-

tivities, hydrolysis and transacylation, as well as the structure of the protein showed a strong dependence upon pH (Casals *et al.*, 1982; Arche *et al.*, 1984; Pérez-Gil *et al.*, 1989b). Moreover, the enzyme inactivation upon chemical modification of essential histidine and carboxy residues is also pH-dependent (Pérez-Gil *et al.*, 1989a, 1990). The aim of the present work is to study the nature of the essential ionizable groups by analysing the effect of pH on the kinetic parameters of lysophosphatidylcholine:lysophosphatidylcholine acyltransferase from rabbit lung. Following the equations derived from the kinetic model previously published (Martín *et al.*, 1990), kinetic parameters other than Michaelis constant and maximum velocity have been evaluated to fit the experimental data.

MATERIALS AND METHODS

Chemicals

1-[1-¹⁴C]Palmitoyl-*sn*-glycero-3-phosphocholine (sp. radioactivity 59 Ci/mol) was purchased from Amersham International (Amersham, Bucks., U.K.). Non-labelled 1-palmitoyl-*sn*-glycero-3-phosphocholine, Hepes, Mes and Bicine were from Sigma Chemical Co. (St. Louis, MO, U.S.A.). All other reagents and solvents were analytical-reagent grade from Merck (Darmstadt, Germany).

Enzyme purification

Lysophosphatidylcholine:lysophosphatidylcholine acyltransferase was purified from rabbit lung by improving the previously described procedure (Casals *et al.*, 1982) by the addition of 10 mM-2-mercaptoethanol to the homogenization buffer in order to preserve higher enzyme specific activity.

Kinetic assays

Both activities of lysophosphatidylcholine:lysophosphatidylcholine acyltransferase, hydrolysis and transacylation, were measured in the same incubation mixture containing 0.1 ml of enzyme solution (0.5 mg of protein/ml), 0.05 ml of substrate

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solution and 0.05 ml of an extended-pH-range buffer. The substrate solution contained 1-[1-¹⁴C]palmitoyl-*sn*-glycero-3-phosphocholine (sp. radioactivity 0.05 Ci/mol) sonicated in distilled water for 30 s at 12 μ M in an MSE sonicator. The buffer was composed of 0.4 M-acetate/0.4 M-phosphate/0.4 M-Bicine and the required pH and ionic strength were achieved by addition of NaOH, HCl and NaCl according to the amounts calculated by the computer program described by Ellis & Morrison (1982). All pH measurements were carried out with a Radiometer 26 pH-meter standardized at the given temperature to a maximum error of ± 0.03 unit by using Beckman standard buffers.

For solvent perturbation experiments, glycerol, dimethyl sulphoxide and dioxan were tested at different concentrations up to 30% (v/v). Dimethyl sulphoxide was chosen because concentrations up to 20% caused a maximum loss of 15% of the control activity, whereas the other two solvents nearly abolished both enzymic activities. Incubations were performed by addition of 0.05 ml of a mixture of dimethyl sulphoxide/water at the adequate proportion to give a final 20% (v/v) dimethyl sulphoxide concentration. A 0.05 ml volume of distilled water was added to the controls. The effect of solvent was tested in both neutral and cationic buffers by using respectively 0.4 M-acetate/0.4 M-phosphate/0.4 M-borate and 0.4 M-Hepes/0.4 M-Mes/0.4 M-Bicine buffers. The organic solvent was added after adjustment of the pH in the assay mixture.

Protein quantification

Protein determination was carried out by a modification of the general Lowry procedure (Ross & Schatz, 1973) in which 2-mercaptoethanol was previously carboxymethylated to prevent absorption at the wavelength of measurement. Horse serum albumin was used as standard.

RESULTS

A kinetic mechanism has been recently proposed for lysophosphatidylcholine:lysophosphatidylcholine acyltransferase that takes into account the special catalytic features of this enzyme: (i) transacylation is a bi-substrate reaction in which both substrates are identical molecules, (ii) an acyl-enzyme covalent intermediate is formed and (iii) the coexistence of hydrolysis and transacylation activities is the result of the competition between water and a second molecule of substrate for the nucleophilic attack on the acyl-enzyme. The initial rates for hydrolysis and transacylation are described by the following theoretical equations (Martín *et al.*, 1990):

$$v_{\text{HD}} = V_{\text{HD}}[S]/([S]^2 + B[S] + A) \quad (1)$$

$$v_{\text{AT}} = V_{\text{AT}}[S]^2/([S]^2 + B[S] + A) \quad (2)$$

where v_{HD} and v_{AT} are respectively the initial velocities of hydrolysis and transacylation. The parameters A , B , V_{HD} and V_{AT} are combinations of the different microscopic kinetic constants and the calculated values were (Martín *et al.*, 1990):

$$A = (1.2 \pm 0.1) \times 10^4 \mu\text{M}^2$$

$$B = 25 \pm 10 \mu\text{M}$$

$$V_{\text{HD}} = (3.50 \pm 0.08) \times 10^4 \mu\text{M} \cdot (\text{nmol/min per mg of protein})$$

$$V_{\text{AT}} = 75 \pm 5 \text{ nmol/min per mg of protein}$$

If the concentration of substrate is low enough, $[S]^2$ and $B[S]$ will be negligible and eqns. (1) and (2) approximate to:

$$v_{\text{HD}} = V_{\text{HD}}[S]/A$$

$$v_{\text{AT}} = V_{\text{AT}}[S]^2/A$$

Under these experimental conditions the parameters V_{HD}/A and V_{AT}/A can be respectively estimated from $v_{\text{HD}}/[S]$ and $v_{\text{AT}}/[S]^2$, and all the binding equilibria are completely displaced

to the free enzyme form. This approach can be considered essentially correct for concentrations of substrate below 25 μ M. Therefore the study of pH-dependence of the parameters V_{HD}/A and V_{AT}/A will provide information about the ionization of essential groups in the free enzyme that are involved in catalysis and/or substrate-binding.

On the other hand, if the substrate concentration is high enough, eqns. (1) and (2) simplify to:

$$v_{\text{HD}} = V_{\text{HD}}/[S]$$

$$v_{\text{AT}} = V_{\text{AT}}$$

Then, v_{AT} and $v_{\text{HD}} \cdot [S]$ are respectively estimators of V_{AT} and V_{HD} . It can be proved that this assumption is essentially true for concentrations of substrate above 400 μ M. In these conditions the enzyme is saturated, the binding equilibria are displaced to the enzyme-substrate complexes and the hydrolysis activity becomes negligible. The concentration of these complexes can be calculated (Martín *et al.*, 1990) as follows:

$$[E \cdot S] = k_{+5}[E]_0/(k_{+2} + k_{+5})$$

$$[F \cdot S] = k_{+2}[E]_0/(k_{+2} + k_{+5})$$

where E and F are free enzyme and acyl-enzyme respectively and k_{+2} and k_{+5} are the kinetic constants of the catalytic steps subsequent to the formation of each complex. The analysis of the pH-dependence of the parameter V_{AT} enables identification of ionizable groups that participate in the acylation of the enzyme from the complex E·S to form F or the deacylation of the enzyme from F·S to transfer the acyl group to the second molecule of substrate.

In accordance with these assumptions, the pH-dependences of the parameters V_{HD}/A and V_{AT} have been studied. Since transacylation and hydrolysis activities are nearly abolished at low and high substrate concentrations respectively, the parameters V_{AT}/A and V_{HD} were not considered.

The plot of parameter V_{HD}/A (estimated from the initial hydrolysis rate for 25 μ M substrate) versus pH is shown in Fig. 1(a). The shape of the curve suggests the existence of an ionizable group responsible for the loss of enzymic activity at acidic pH (Tipton & Dixon, 1979). The ionization pK for this group can be calculated by fitting the experimental data to the equation proposed by Cleland (1982):

$$Y = Y_0/(1 + 10^{(pK - pH)}) \quad (3)$$

where Y is the kinetic parameter measured and Y_0 is the asymptotic value of Y at high pH. Calculation was made using the non-linear-regression facility of the statistical software package BMDP (Dixon, 1981) and the calculated pK at 37 °C was 5.66 ± 0.06 . The plot of V_{AT} versus pH (as initial transacylation rate for 500 μ M substrate) behaved in a similar way (Fig. 1b) and the pK value at the same temperature was 5.74 ± 0.06 . In both cases the asymptote at low pH had a limiting slope of +1, indicating that protonation of a single residue is responsible for the detected pK.

Further evidence on the nature of the essential ionizable groups in the enzyme was obtained by examining the effect on pK values of a decrease in the polarity of the solvent by addition of water-miscible organic solvents (Tipton & Dixon, 1979). The effect of 20% (v/v) dimethyl sulphoxide on pK is shown in Table 1. In cationic acid buffer no change was observed in the pK calculated from the V_{HD}/A -pH profile, whereas in neutral acid buffer the pK significantly decreased. The deprotonation of a cationic acid group in neutral acid buffer involves the disappearance of charged species, whereas in cationic acid buffer no change of total charge exists. Since lowering the dielectric constant favours uncharged species, organic solvent is expected

Fig. 1. Plots of kinetic parameters (a) V_{HD}/A and (b) V_{AT} versus pH.

0.4 M-Acetate/0.4 M-phosphate/0.4 M-Bicine was used as wide-pH-range buffer. Samples contained 0.1 ml of enzyme solution, 0.05 ml of substrate solution and 0.05 ml of buffer, and were incubated for 1 min at 37 °C.

Table 1. Effect of dimethyl sulphoxide on pK values calculated from $\log(V_{HD}/A)$ -pH and $\log(V_{AT})$ -pH profiles, in either neutral or cationic acid buffers

Kinetic parameters V_{HD}/A and V_{AT} were calculated at different pH values in the presence (+ Me₂SO) or in the absence (- Me₂SO) of 20% (v/v) dimethyl sulphoxide. The neutral and cationic acid buffers were respectively 0.4 M-acetate/0.4 M-phosphate/0.4 M-borate and 0.4 M-Mes/0.4 M-Hepes/0.4 M-Bicine. pK values and their standard deviations were obtained by statistical analysis of the parameter data in accordance with eqn. (3).

Buffer type	$\log(V_{HD}/A)$		$\log(V_{AT})$	
	- Me ₂ SO	+ Me ₂ SO	- Me ₂ SO	+ Me ₂ SO
Neutral	5.68 ± 0.12	5.34 ± 0.18	5.26 ± 0.18	5.18 ± 0.18
Cationic acid	5.61 ± 0.12	5.62 ± 0.15	5.44 ± 0.15	5.65 ± 0.14

to decrease the pK of the group in the former type of buffer and not to affect in the latter one. Thus the group responsible of the observed pK should be a cationic acid residue. As lysophosphatidylcholine does not contain any ionizable group at this pK, it follows that the deprotonation of a residue at the active site of the enzyme is required for catalytic activity. This conclusion cannot be confirmed from the V_{AT} -pH profile because, as mentioned above, the pK deduced from this parameter corresponds to enzyme-substrate complexes and the effect of the solvent polarity on the ionizable groups in these complexes is more difficult to evaluate.

The temperature-dependence of V_{HD}/A -pH and V_{AT} -pH profiles was examined in order to calculate the ionization enthalpy of the group whose protonation causes the loss of enzyme activity. Experiments were carried out at 22, 30, 38, 43 and 49 °C

Fig. 2. van't Hoff plots of pK values obtained for the kinetic parameters (a) V_{HD}/A and (b) V_{AT}

Experiments similar to those of Fig. 1 were carried out at five temperatures, and the corresponding pK values were calculated. Enthalpies of ionization were calculated by weighed least-squares fitting of the slopes.

(Figs. 2a and 2b). No irreversible thermal inactivation was observed in these experimental conditions. Fitting the data to the van't Hoff equation enabled us to obtain the following enthalpies:

$$\Delta H_{ion} = -54 \pm 4 \text{ kJ/mol, from } V_{HD}/A$$

$$\Delta H_{ion} = -76 \pm 5 \text{ kJ/mol, from } V_{AT}$$

Taking into account that there are no residues in proteins with so negative an ionization enthalpy (Cleland, 1977), this high exothermic enthalpy could be assigned to a conformational change concomitant with the deprotonation of the essential residue.

DISCUSSION

The analysis of the effect of pH on the catalytic properties of enzymes is a very useful tool for gaining information about their catalytic mechanisms. Previous studies have reported several pH-dependent events in lysophosphatidylcholine:lysophosphatidylcholine acyltransferase. Both activities of the enzyme change more steeply at acidic than at alkaline pH (Casals *et al.*, 1982). The secondary structure of the protein showed a similar behaviour with respect to pH changes to that deduced from far-u.v. c.d. studies (Arche *et al.*, 1984). The maintenance of the active structure of the enzyme was shown to be critically dependent on the ionization of a group with pK 5.8. We have suggested that this group could be particularly involved in the stabilization of the specific geometry of the active site (Pérez-Gil *et al.*, 1989b). Chemical modification of lysophosphatidylcholine:lysophosphatidylcholine acyltransferase by specific reagents pointed to the existence of essential histidine residues at the active site of the enzyme (Pérez-Gil *et al.*, 1989a). Furthermore, a histidine residue was tentatively assigned to the group responsible for the structural change at acidic pH (Arche *et al.*, 1984). Nevertheless, none of these pieces of evidence could be directly related to

catalysis until the pH-dependence of the catalytic mechanism had been investigated.

The kinetics of the reaction catalysed by lysophosphatidylcholine:lysophosphatidylcholine acyltransferase could not be analysed until a kinetic mechanism was proposed and demonstrated (Martín *et al.*, 1990). The novelty of the non-Michaelian kinetic behaviour that was derived from the model made it necessary to seek new parameters that permit the study of certain enzyme properties such as reversible inhibition patterns and essential ionization equilibria in catalytic mechanism.

The V_{HD}/A -pH profile (Fig. 1a) led to a pK value of 5.66. This result suggests that the deprotonation of one ionizable group in the free enzyme is essential in the overall catalytic mechanism. The V_{AT} -pH profile (Fig. 1b) showed a pK of 5.74, indicating that the substrate-enzyme complexes require the deprotonation of one residue of the protein for catalysis. Since two complexes, E·S and F·S, can exist at saturating substrate concentration, the pK value obtained cannot be unequivocally assigned to only one of the enzyme species. However, the great accuracy of the fitting to eqn. (3) suggests that either the pK values are identical in both existing complexes or only one of them is accumulated under saturation conditions owing to the existence of a rate-limiting step. On the other hand, the similarity of the two reported pK values suggests that the same ionizable group is observed in both cases. It follows that this essential residue is directly participating in one of the steps in the chemical mechanism of catalysis, i.e. acylation or deacylation of the enzyme. Although the involvement in binding cannot be completely ruled out, it is unlikely because ionization does not seem to be perturbed by substrate-binding.

As pointed out by Cleland (1977), an observed pK around 5.7 should correspond to two types of groups: (i) a histidine residue with lowered pK due to the formation of an ionic pair or (ii) either a histidine or a carboxy residue buried into a hydrophobic environment. In order to discriminate between histidine and carboxy residues, the influence of organic solvents on pK and the values of the enthalpies of ionization were studied. The results in Table 1 point to a histidine residue as the responsible group of this pK and this conclusion agrees with previous studies on chemical modification of the enzyme (Pérez-Gil *et al.*, 1989a). The highly exothermic values of the enthalpies of ionization that are derived from both V_{HD}/A and V_{AT} are remarkable. The expected values for histidine and carboxy residues are 29 and 0 kJ/mol respectively (Cleland, 1977), and no amino acid residue in proteins has been reported with so negative an enthalpy.

Therefore this result points to the existence of a thermodynamically favourable conformational re-arrangement in the protein structure that is concomitant with the deprotonation of the essential histidine residue. This conclusion agrees with the previously reported existence of a conformational transition that depends on a pK around 5.5, as observed in the far-u.v. c.d. spectra of the enzyme (Arche *et al.*, 1984). Then, the protonation of a histidine residue at the active site would have two consequences: (i) the conversion of the enzyme into a catalytically non-competent form and (ii) the appearance of new interactions at the active-site microenvironment giving a conformational transition.

A recent paper (Pérez-Gil *et al.*, 1990) has reported the involvement of essential cysteine residues, but no serine, at the

active site of lysophosphatidylcholine:lysophosphatidylcholine acyltransferase from rabbit lung. Taking this fact into account, a chemical mechanism for the reaction catalysed by this enzyme can be proposed. The acyl-enzyme, the key intermediate for the kinetic mechanism, would originate from the nucleophilic attack on the substrate molecule by a cysteine residue located at the active site. The nucleophilicity of the catalytic cysteine residue should be enhanced by ionic pairing with an imidazole group, as occurs in other related enzymes (Estrada *et al.*, 1985). This mechanism of activation of the catalytically essential thiol group by cysteine-histidine pairing is well known in other acyl-transfer mechanisms, e.g. cysteine proteinases (Polgár, 1973) and cysteine β -lactamases (Knap & Pratt, 1989), which also follow ping-pong kinetics and require the activation of the primary nucleophile of the active site by an adjacent functional group.

Proof of the existence of a thioester acyl-enzyme will require more sophisticated kinetic studies. However, the present results not only provide additional evidence on the catalytic mechanism of lysophosphatidylcholine:lysophosphatidylcholine acyltransferase, but also confirm the kinetic model previously reported for this important enzyme of the mammalian lung.

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