Substrate interferes with dimerisation of outer membrane phospholipase A

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Abstract Outer membrane phospholipase A (OMPLA) activity is regulated by reversible dimerisation with the dimer being the active species. Observed lag phases in activity indicated that dimerisation may be slow relative to turnover. A covalent OMPLA dimer indeed did not display lag phase behaviour. A model for OMPLA kinetics was proposed accounting for a slow dimerisation step. Preincubation conditions determined the initial amount of monomer and influenced both lag times and final activities. Under the conditions used, substrate concentrations higher than 50 mol% inhibited OMPLA activity and increased lag times. Our results may shed more light on mechanisms controlling OMPLA activity in vivo.

Key words: Outer membrane phospholipase A; Membrane enzyme; Kinetics; Lag phase; Dimerisation

1. Introduction

The outer membrane phospholipase A (OMPLA) is an integral membrane enzyme that catalyses the hydrolysis of acyl ester bonds in phospholipids using calcium as a cofactor [1]. The enzyme is present in many pathogenic and non-pathogenic Gram-negative bacteria. In pathogenic bacteria such as *Campylobacter coli* or *Helicobacter pylori* OMPLA is involved in pathogenesis and virulence [2,3], but its role in non-pathogenic bacteria is less clear. In *Escherichia coli* OMPLA is involved in the secretion of colicins [4,5], but since OMPLA is also present in strains that do not harbour the plasmid-encoded colicins, it is unlikely that this is the main function of the enzyme.

In the outer membrane environment all ingredients for OMPLA catalysis are present, i.e. the enzyme, phospholipids and calcium, yet no active membrane phospholipid turnover is detected under physiological conditions [6]. The need for a tight regulation of OMPLA activity is obvious as uncontrolled OMPLA activation would be lethal to the cell. Activation of OMPLA can be induced by processes that disturb the outer membrane integrity [5,7,8]. This activation coincides with dimerisation, suggesting that dimerisation is important for activity regulation [9]. Structural studies revealed that only upon dimerisation functional active sites, substrate binding pockets and calcium sites are formed [10]. The calcium site is liganded by residues from both monomers and is absolutely required for enzymatic activity [11,12]. In a micellar system high affinity calcium binding is strictly correlated with dimerisation. However, in a phospholipid bilayer dimerisation is not sufficient for catalysis; the physical state of the bilayer and the integrity of the dimer interface are also important (unpublished results).

To further understand regulation of OMPLA activity by the substrate and assay conditions, this study focuses on the origin of kinetic lag phases encountered during OMPLA catalysis.

2. Materials and methods

2.1. Chemicals

Research grade dodecyl-*N*,*N*′-dimethyl-1-ammonio-3-propanesulphonate (C_{12}SB) was obtained from Fluka and purified as described before [13]. 2-Hexadecanoylthio-ethane-1-phosphocholine (C_{16}thioglycol*P*Cho) was synthesised according to Aarsman et al. [14]. All other chemicals were of the highest purity commercially available.

2.2. Native OMPLA

OMPLA was overproduced in BL21(DE3) containing the plasmid pRK21 after induction with isopropyl-β-D-1-thiogalactopyranoside. Subsequent isolation of inclusion bodies, folding and purification were carried out essentially as described by Dekker et al. [13].

2.3. Construction of a covalent OMPLA dimer

OMPLA containing a His26Cys mutation was employed for the construction of a covalent OMPLA dimer. His26Cys OMPLA was expressed from plasmid pND5 [15] and purified in the presence of 5 mM dithiothreitol (DTT). To free the protein from DTT, it was loaded onto a Q-Sepharose column, washed with buffer A (2.5 mM C_{12}SB, 20 mM Tris–HCl, pH 8.3, 20 mM $CaCl_2$) and subsequent elution with 1 M KCl in buffer A. The protein was incubated overnight for optimal disulphide bond formation. The dimeric species of OMPLA was further purified by application onto a Superdex G-200 column (Pharmacia).

2.4. Activity assay

Routinely, OMPLA activities were measured spectrophotometrically using C_{16}thioglycol*P*Cho as a substrate. OMPLA was preincubated for 2 h at 0.05 mg/ml in 20 mM Tris–HCl, pH 8.3, 2 mM EDTA, pH 8.0, 5 mM C_{12}SB. 50 ng of protein was assayed for enzymatic activity in 1 ml of assay buffer (50 mM Tris–HCl, pH 8.3, 5 mM $CaCl_2$, 0.2 mM Triton X-100, 0.1 mM dithiobis(2-nitrobenzoic acid), 0.25 mM substrate). Velocities were calculated from the increase in absorbance at 412 nm.

Several parameters were varied in this assay to study OMPLA

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Abbreviations: C_{12}SB, dodecyl-*N*,*N*-dimethyl-1-ammonio-3-propanesulphonate; C_{16}thioglycol*P*Cho, 2-hexadecanoylthio-ethane-1-phosphocholine; DTT, 1,4-dithiothreitol; OMPLA, outer membrane phospholipase A

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kinetics, i.e. the concentration of $C_{12}SB$ in the preincubation and the concentrations of Triton X-100 and substrate in the assay.

For the determination of apparent calcium binding constants in the kinetic assay, $CaCl_2$ in the assay buffer was replaced by 10 μM EDTA and the enzymatic activities were measured after addition of various calcium concentrations to the assay buffer. The binding parameters were determined by non-linear regression.

3. Results and discussion

3.1. Kinetic observations

OMPLA is a rather non-specific acyl hydrolase that cleaves the ester bonds of long chain fatty acids from the glycerol backbone of phospholipids at both the *sn*-1 and *sn*-2 position irrespective of the polar head group present [1]. To simplify OMPLA kinetics, the monoacyl substrate C_{16}thioglycol*P*Cho was used.

Routinely, OMPLA was preincubated with 2.5 mM of the detergent $C_{12}SB$ in the absence of calcium. At modest concentrations (e.g. 20 mol%) of the substrate C_{16}thioglycol*P*Cho in 0.2 mM Triton X-100 identical reaction rates were obtained when either OMPLA or substrate was added first to Triton X-100 micelles (data not shown). However, a long lag phase was observed when OMPLA was added to 200 μM Triton X-100 micelles containing 70 mol% of substrate (Fig. 1, trace A). In contrast, zero order kinetics were obtained when enzyme was added first to 200 μM Triton X-100 followed by the addition of substrate (Fig. 1, trace B). In both cases the incubation mixture contained the essential cofactor calcium at 5 mM. When substrate and OMPLA were allowed to equilibrate by standing overnight in the absence of calcium, lag phases were noted irrespective of the order of addition of enzyme and substrate.

A first indication of slow monomer to dimer equilibration of OMPLA was obtained, when it was observed that a covalent OMPLA dimer was fully active under all conditions showing zero order kinetics only.

3.2. Kinetic model

Based on these observations we propose a simple model for OMPLA kinetics (Fig. 2). The first step in this model describes the (slow) formation of a catalytically competent dimer from two inactive monomers. Once this dimer is formed, substrate is rapidly converted into product. A consequence of slow dimer formation is also slow dissociation of dimeric OMPLA formed. A mathematical description of the proposed model is essentially identical to the kinetic treatment of the action of phospholipase A2 as described by Verger et al. [16]. The amount of reaction product (*P*) formed with time (*t*) was fitted to:

$$P = at + ab(\exp^{-t/b} - 1) \quad (1)$$

Final enzymatic activity (dP/dt) is represented by constant *a*, while constant *b* describes the lag time of the equilibrium $M \Leftrightarrow 0.5$ D. The active sites in the dimer are treated as independent, such that only one active site is considered in 0.5 D. The activity curves correlated well with this equation, with the correlation coefficient being above 0.99 in all cases.

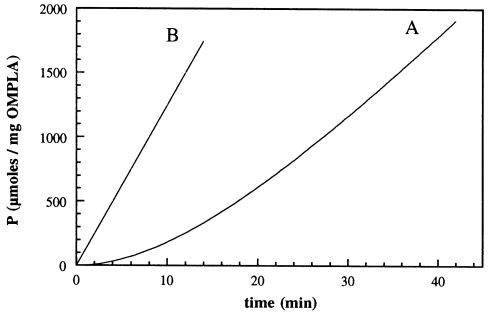

Fig. 1. Activity curve of native OMPLA under lag phase conditions (A) and non-lag phase conditions (B). A: OMPLA was preincubated at 0.05 mg/ml in 10 mM $C_{12}SB$ and 50 ng was tested for its activity in mixed substrate/Triton X-100 micelles (71 mol% of substrate at a Triton X-100 concentration of 200 μM). B: OMPLA was preincubated at 0.05 mg/ml in 10 mM $C_{12}SB$ and 50 ng was added to assay mix containing 200 μM Triton X-100 prior to addition of 500 μM substrate.

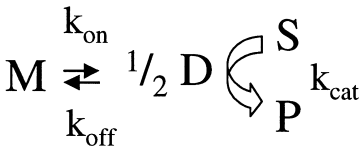

Fig. 2. Model for OMPLA catalysis. Only upon formation of a dimer two functional active sites and substrate binding pockets are formed that are assumed to act independently. Monomeric OMPLA (M) associates to yield a catalytically competent dimer (D). The dimerisation equilibrium is strongly influenced by experimental conditions such as type of detergent, concentration of detergent and substrate concentration. Once a dimer is formed, substrate (S) is rapidly degraded to yield product (P).

3.3. Influence of assay parameters on lag phases and enzymatic activity

Previously, it was shown that the experimental parameters strongly determine OMPLA activity [9]. Our current activity model Eq. 1 was used to further study the influence of assay parameters on both the occurrence of lag phases and final OMPLA activity and hence the conditions required for OMPLA dimerisation. A better understanding of OMPLA catalysis in vitro may lead to an increased understanding of OMPLA action within the cellular context.

Previous work demonstrated that excess of the detergent $C_{12}SB$ leads to dissociation of dimeric OMPLA and subsequent loss in apparent initial enzymatic activity [9]. Thus, after preincubation at 20 mM $C_{12}SB$ only 10% residual initial activity was retained. However, longer incubation times revealed the presence of lag phases at elevated detergent levels. Fig. 3 shows final OMPLA activities and lag phases as a function of the concentration of the zwitterionic detergent $C_{12}SB$ during preincubation. Final OMPLA activity decreased with increasing $C_{12}SB$ concentrations, but only modestly. At 20 mM $C_{12}SB$ only 50% of the final OMPLA activity was lost. A strong increase in lag times was observed as well at higher $C_{12}SB$ concentrations, explaining the discrepancy with earlier work [9]. The lag time increased linearly with $C_{12}SB$ concentration with a slope of 1.7 min/mM $C_{12}SB$. The intercept on the *x*-axis was found to be 1.2 mM $C_{12}SB$ which is close to the critical micelle concentration of $C_{12}SB$ (at 1.3 mM).

The effect of $C_{12}SB$ concentration was not seen when preincubated enzyme was added to Triton X-100 micelles in the assay prior to substrate, suggesting that under these conditions catalytically competent OMPLA dimers are formed that readily bind and convert substrate and only slowly dis-

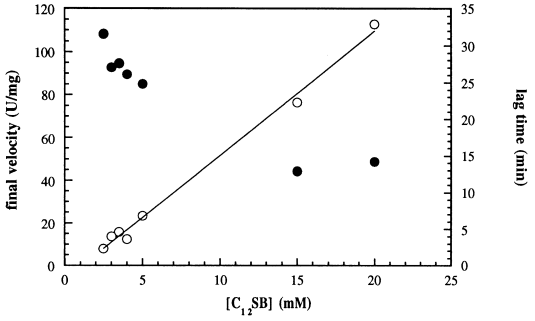

Fig. 3. Influence of detergent concentration in the preincubation on OMPLA catalytic turnover and lag times. OMPLA was preincubated at variable concentration of C_{12}SB at 0.05 mg/ml. 50 ng of protein was assayed in 500 μM substrate, 200 μM Triton X-100 (71 mol% substrate). Final velocities (●) and lag times (○) were determined using Eq. 1.

sociate. Lag phases were also not observed for a covalent OMPLA dimer (data not shown), confirming this hypothesis.

Calcium is absolutely required for OMPLA activity and also stimulates dimerisation of the enzyme. Initial OMPLA activities at various calcium concentrations could be fitted to a hyperbolic saturation curve to yield a calcium affinity of 18 μM [17]. The final OMPLA activities obtained with Eq. 1 were fitted identically as initial OMPLA activities to obtain an affinity constant of 11 μM. Lag times were found to increase at lower calcium concentrations, indicating that at lower calcium concentrations dimers are formed only slowly.

Whereas the importance of calcium and detergent in the dimerisation of OMPLA has previously been studied, the role of substrate in the dimerisation of the enzyme was less clear, but is particularly relevant because in vivo the enzyme is embedded in its substrate. Previously, it was shown that low substrate concentrations promoted dimerisation [15]. In the present work the kinetics of OMPLA hydrolysis were investigated at high substrate concentrations in an attempt to mimic the in vivo situation.

At 200 μM of Triton X-100, up to 20 mol% substrate a hyperbolic saturation curve was obtained from which the affinity for the substrate was derived. The substrate affinity constant K^*_m was found to be 11 mol% C_{16}thioglycol*P*Cho. In Fig. 4A the influence of high molar fractions of substrate on OMPLA activity is shown. Maximum activity was observed around 40–50 mol% of substrate. This maximum did not depend on the total concentration of Triton X-100 in the assay when varied from 200 to 800 μM. The data were fitted to a model describing inhibition of OMPLA by several molecules of substrate:

$$v = \frac{v_{max}}{1 + \frac{K^*_m}{S} + \left(\frac{S}{K_i}\right)^n} \quad (2)$$

with v being catalytic turnover (U/mg), v_{max} the maximum catalytic turnover (U/mg), K^*_m the affinity for the substrate (mol%), S the concentration of substrate (mol%), K_i the inhibition constant (mol%) and n the number of substrate molecules involved in the inhibition of one OMPLA active site. The affinity constant K^*_m for the substrate was derived from experiments at a low molar fraction of substrate, being 11 mol%. Fitting experimental data to Eq. 2 yielded a v_{max} of 170 ± 6 U/mg, a K_i of 66 ± 1 mol% and an n of 6 ± 1. Also lag times were strongly affected by the molar fraction of substrate and increased linearly with increase in molar fraction of substrate, regardless of the concentration of Triton X-100 and substrate used (Fig. 4B). The increase in lag time was found to be 0.24 min/mol% of substrate, and the intercept with the x-axis yielded 3 mol%.

Zero order behaviour was observed when either 250, 500 or 1000 μM substrate was added to 200 μM Triton X-100 micelles containing OMPLA, suggesting that under these conditions the enzyme was present as a pre-formed dimer. At elevated concentrations of Triton X-100 lag phases were observed, indicating that excess Triton X-100 hindered the association of OMPLA into pre-formed catalytically competent dimers.

Thus, it was found that rapid hydrolysis occurred when substrate was added to OMPLA that was dimeric either as a pre-formed or covalently bound species. Apart from the concentration of C_{12}SB during preincubation, also substrate was found to be inhibitory to OMPLA activity. Twelve substrate molecules were found to be involved in the dissociation of a non-covalent OMPLA dimer. The relevance of substrate inhibition by dissociation of the catalytically active species of OMPLA in vivo is not obvious. However, a function for OMPLA as housekeeping enzyme has been suggested. In such a role, its activity should be tightly controlled. The inhibition of OMPLA by its substrate may well provide the means to prevent OMPLA activity from being lethal to the cell once activated. Further investigations will be required to confirm a role for the substrate in OMPLA activity regulation in vivo.

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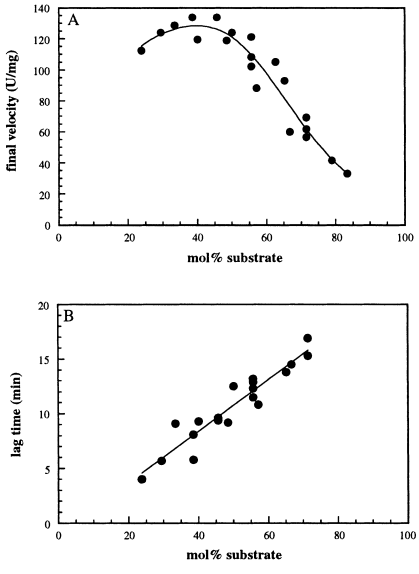

Fig. 4. Influence of substrate concentration on OMPLA catalytic turnover and lag times. A: Dependence of final OMPLA activity on substrate at high concentrations. B: Influence of substrate concentration on lag times. The enzymatic activity of OMPLA was determined after preincubation at 10 mM C_{12}SB. Triton X-100 was varied between 200 and 800 μM and substrate concentration was varied between 250 and 1000 μM to yield a variety of substrate concentrations. The data of panel A were fitted to Eq. 2.

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