THE PREPARATION OF ACTIVATED BEE VENOM PHOSPHOLIPASE A_2

Denis DRAINAS, Geoffrey R. MOORES and Anthony J. LAWRENCE
Department of Cell Biology, The University of Glasgow, Glasgow G12 8QQ, Scotland

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1. Introduction

Many phospholipase enzymes are activated by long-chain fatty acids, but the sensitivity to activation depends on the nature of the substrate. It therefore has been concluded that fatty acids intercalate into the lipid phase and modify the primary interaction of the enzyme with its substrate [1]. Bee venom phospholipase A_2 is activated by fatty acids [2], but the kinetics of activation do not support this substrate-mediated mechanism (unpublished data); however the high affinity of both substrate and enzyme for free long-chain fatty acids makes investigation by kinetic means both difficult and unreliable.

This enzyme can be activated irreversibly by treatment with acid anhydrides presumed to add long-chain acyl residues to nucleophilic groups in the protein [2]. If all of the effects of fatty acid activation were produced by covalent addition of a single acyl residue, fatty acid activation could be attributed to allosteric properties of the enzyme. It would then be feasible to design reagents for selectively activating or blocking the activation of this and related enzymes in vivo. Lauroyl ethyl carbonate (the mixed anhydride of lauric acid and ethyl carbonic acid) was chosen as activator for this study because it gave rapid activation with little non-specific inactivation (tested against a non-activating substrate) and free lauric acid is a relatively weak activator at corresponding concentrations. Dimethyl maleic anhydride, a reversible blocking agent for amino-groups [3] was used initially to protect the protein against non-specific acylation, but its direct action on fatty acid activation became of greater interest here.

2. Methods and materials

Reaction rates were measured by conductimetry [4,5] at 37°C in 10 mM triethanolamine/Cl^- buffer, pH 8.2, containing 0.1 mM $CaCl_2$ and prepared in 20%, v/v, *n*-propanol/water (reaction buffer). The response was linear over the range 0–1 mM oleic acid. Chemical modifications of the enzyme were carried out with protein (1 mg/ml) in reaction buffer and activators or inhibitors were added from stock 2%, w/v, solutions in acetone. The triethanolamine concentration was increased to 100 mM for treatments involving high concentrations of dimethyl maleic anhydride. Gel filtration was Bio-gel on P-6 (Bio Rad)/ packed in a column (12 × 105 mm) and equilibrated and eluted with reaction buffer.

Purified bee venom phospholipase A_2 [6] was a gift from Dr R. Shipolini (Univ. Coll. London). Glycerophosphoryl choline was prepared by the method in [7] and reacylated by the method in [8]. The products, dioleoyl and diundecenoyl phosphatidyl choline were purified by chromatography on alumina, deionised using Zerolite DMF resin and were chromatographically homogenous on thin-layer chromatography.

Lauroyl ethyl carbonate (LEC) was prepared from potassium laurate and purified ethyl chloroformate (B.D.H.) [9]. The product was characterised by infrared analysis. ^{14}C-Labelled LEC was prepared from [^{14}C]lauric acid (The Radiochemical Centre, Amersham) at spec. act. 3.3 μCi/μmol. Dimethyl maleic anhydride (DMMA) and fatty acids were from Sigma Chemical Co.

3. Results

The hydrolysis of dioleoyl phosphatidyl choline by bee venom phospholipase A_2 is biphasic due to product activation by fatty acid [2]. Monophasic curves are produced by addition of free lauric acid, fig.1. Lauric acid is a stronger activator than the produce oleic acid, but higher concentrations are required for maximum effect (1 mm cf. 0.05 mM). The hydrolysis of diundecenoyl phosphatidyl choline is monophasic and weakly inhibited by lauric acid.

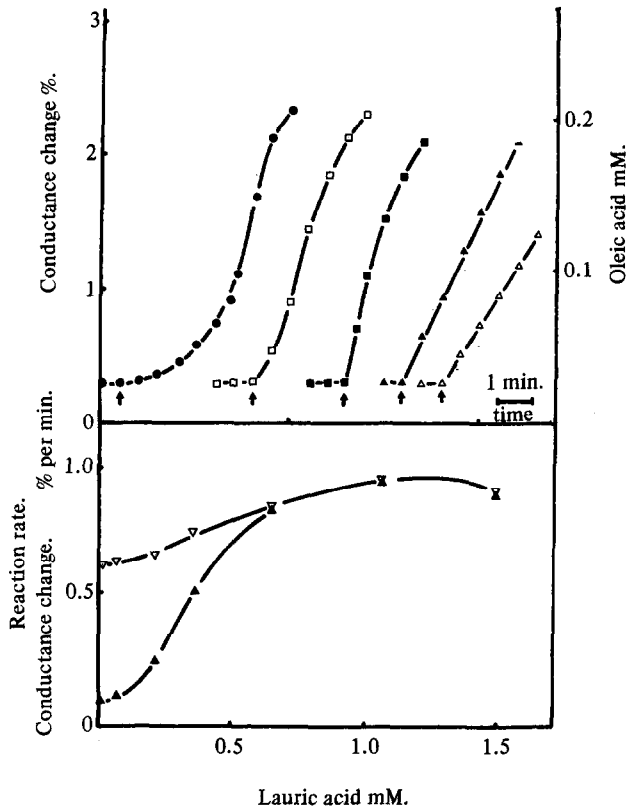

Fig.1a. The hydrolysis of phosphatidyl choline derivatives by bee venom phospholipase A_2 (2.5 μg per ml) at 37°C, followed by conductimetric assay in 1 ml 10 mM triethanolamine/Cl^- buffer, pH 8.2, containing 0.1 mM $CaCl_2$ and 20%, v/v, *n*-propanol. Dioleoyl phosphatidyl choline substrate (0.49 mM), with (●), (□), (■), 0, 0.22 and 0.66 mM lauric acid respectively. Diundecenoyl phosphatidyl choline substrate (0.95 mM) with (▲), (△) 0 and 0.66 mM lauric acid, respectively. Fig.1b. The hydrolysis of dioleoyl phosphatidyl choline was followed as above and the initial and maximum rates of hydrolysis plotted against lauric acid concentration.

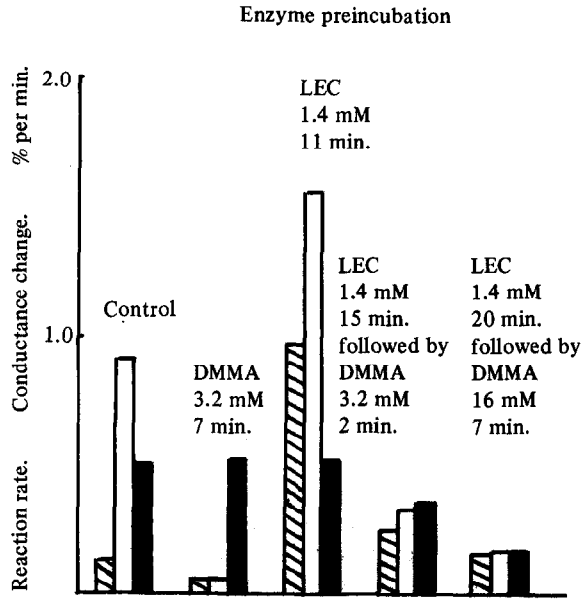

Fig.2. The initial and maximum rates of hydrolysis of dioleoyl phosphatidyl choline and the initial rate of hydrolysis of diundecenoyl phosphatidyl choline were measured as in fig.1. The enzyme (1 mg/ml in propanolic reaction buffer) was preincubated with lauroyl ethyl carbonate (LEC) and/or dimethyl maleic anhydride (DMMA) and 2.5 μl solution assayed in 1 ml substrate solution. (□) Dioleoyl phosphatidyl choline, initial rate. (▩) Dioleoyl phosphatidyl choline, maximum rate. (■) Diundecenoyl phosphatidyl choline, initial rate.

Preincubation of the enzyme with lauroyl ethyl carbonate (LEC) in the absence of substrate progressively eliminates the biphasic curve for the long-chain substrate, but has no effect with the short-chain substrate, fig.2. Preincubation with dimethyl maleic anhydride (DMMA) has the same result but acts by inhibiting the late phase of hydrolysis of dioleoyl phosphatidyl choline rather than activating the early phase. Enzyme activated with LEC and then treated with DMMA retains the nearly linear curve for hydrolysis of dioleoyl phosphatidyl choline, but activity is reduced at least 5-fold. This treatment also reduces the activity towards diundecenoyl phosphatidyl choline and at higher concentrations of DMMA, hydrolysis of both substrates is monophasic at the same reaction rate.

Activation by LEC cannot be ascribed to free lauric acid, because controls in which LEC was replaced

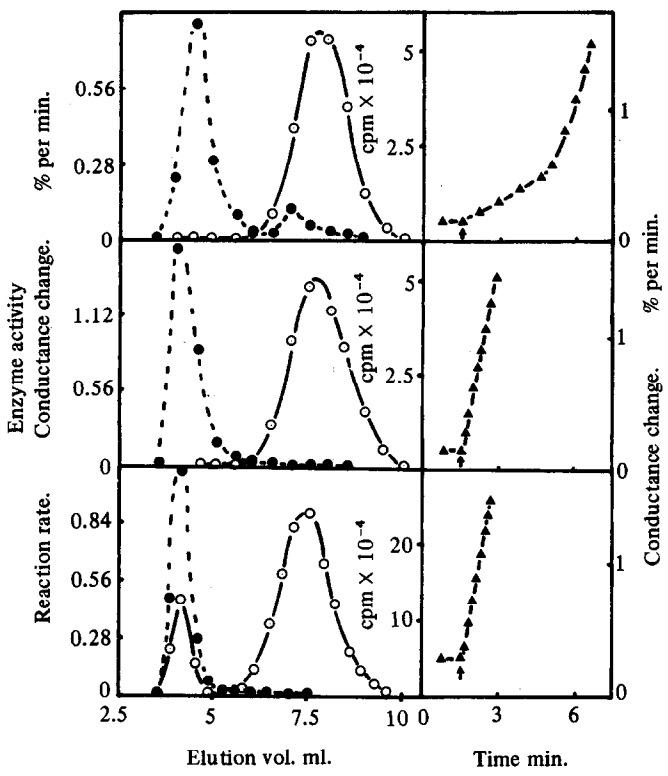

Fig.3. Solution, 250 μl, bee venom phospholipase A_2 (1 mg/ml reaction buffer; approx. 0.016 μmol. enzyme) was passed through a column (12 × 105 mm) of Bio-Gel P-6 equilibrated in this buffer. (a) After addition of 0.18 μmol [^{14}C]lauric acid; spec. act. approx. 14 μCi/μmol. (b) After incubation with 0.18 μmol lauroyl ethyl carbonate for 12 min followed by addition of [^{14}C]lauric acid as above. (c) Enzyme solution, 500 μl, was preincubated with 0.36 μmol of [^{14}C]lauroyl ethyl carbonate; spec. act. 3.4 μCi/μmol. Enzyme activities were determined using diundecenoyl phosphatidyl choline as substrate (•). In each case enzyme from the most active fraction was tested for activation using dioleoyl phosphatidyl choline substrate (▲). Fractions, 400 μl (a) and (b) and 300 μl (c) were added to 10 ml Aquasol scintillant for ^{14}C determination (○).

by the equivalent concentration of lauric acid showed no activation. Definitive proof was obtained using gel filtration to separate unactivated enzyme and enzyme activated by both 'cold' and ^{14}C-labelled LEC, from tracer lauric acid or LEC, fig.3a–c.

The activated enzyme retains full activation after the treatment and is stable in this form indefinitely. Enzyme activated with ^{14}C-labelled LEC became labelled corresponding to uptake of 1.8 lauroyl residues/molecule.

4. Discussion

These preliminary experiments prove that acylating agents which activate bee venom phospholipase A_2 do so by modifying the enzyme and not by releasing fatty acids which then interact with the substrate. Activation may tentatively be ascribed to the addition of a single acyl residue to the protein molecule resulting in an allosteric modification which can be sensed by long-chain but not by short-chain substrates. The results imply that activation by free fatty acids involves the same mechanism.

The labelling studies show that long-chain acylating agents are highly specific for the activating site (attacking 2 out of approx. 20 possible nucleophilic residues [10]). In this respect acyl ethyl carbonates are superior to acid chlorides or anhydrides. Specificity is increased by increasing the side-chain length and the lauroyl residue was chosen for this study only to minimise direct activation by free acid. Therefore there is no doubt that a range of highly specific activators can be prepared for this enzyme and possibly for related enzymes.

The action of dimethyl maleic anhydride on untreated enzyme shows that activation can be blocked without generally inactivating the enzyme, fig.2, but in contrast it gives general inactivation for covalently activated enzyme.

In summary therefore: chemical means are available to activate bee venom phospholipase A_2 to block activation and to 'reverse' the effects of activators even after they are bound to the protein.

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