ACTIVATION OF BEE VENOM PHOSPHOLIPASE A_2 BY FATTY ACIDS, ALIPHATIC ANHYDRIDES AND GLUTARALDEHYDE

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1. Introduction

The phospholipase from Bee venom, a small cationic protein (mol. wt 14 629, pI 10.5 ± 1.0) of known primary structure [1,2] hydrolyses long-chain phosphatidyl phospholipids most rapidly in dilute aqueous solutions of organic solvents (e.g. *n*-propanol) where it shows product activation [3] which becomes more pronounced as the solvent concentration is decreased. Activation of lipases is a common phenomenon of possible importance in many regulatory processes [4,5] and the explanations proposed concentrate on possible modification of the substrate by the activator to increase attraction to, or penetration of the surface by the enzyme [6,7]. The Bee venom enzyme is activated by fatty acid anions (e.g. palmitate, oleate) with a minor contribution from lysolecithin, fig. 1a, and both products are inhibitory although this is masked at low concentrations. The kinetics of the system are deceptively simple, an individual progress curve having a near linear slow phase followed after a brief transition by a fast, activated phase. Whilst the rate in the activated phase is highly dependent on substrate concentration, that in the slow phase is not, showing that activation, which increases V_{max} approx. 25-fold appears to decrease affinity for the substrate. This result is more readily explained (given in detail in a later paper) by postulating that activators modify the enzyme not the substrate, a conclusion strongly reinforced by the experiments presented here.

2. Materials and methods

Phosphatidyl-choline was purified from egg yolk by the methods of Ansell and Hawthorne [8] and further freed from fatty acid by passage through a short column of mixed bed ion exchange resin in 1:1, v/v *n*-propanol/water. Purified bee venom phospholipase A_2 was a gift from Dr R. Shipolini [1] (University College, London). Reactions were followed by conductimetry [3,9] with continuous recording from six 1.5 ml cells (10 mm ID), using a solution of *n*-propanol/water 1:4 v/v buffered to pH 8.2 with 10 mM triethanolamine-isobutyrate containing 0.1 mM Ca^{2+}. Glutaraldehyde was from Sigma Chemical Co. Ltd. and used as a 10% solution in water. Butyric and acetic anhydrides were from B.D.H. Ltd. and used as 2.5% solutions in ether. Decanoic anhydride was prepared from decanoic acid by treatment with dicyclohexyl carbodimide, [10] and used as a solution in ether. Enzyme was preincubated in assay buffer and treated with amino group reagents in the presence or absence of oleic acid. Samples were diluted 750-fold for assay to give negligeable carry-over of activator. Anhydrides were extracted from enzyme samples with petroleum ether and controls showed no loss of enzyme activity.

3. Results and discussion

Incubation of the enzyme with dilute glutaraldehyde appeared to destroy activatability without greatly reducing initial activity, whilst in the presence of oleic acid this treatment rapidly stabilised activity at a much higher level again reducing activatability, fig. 1b. This could be due to stabilisation by cross-linking of low activity and high activity conformations of the enzyme, indicating that fatty acid activators

cause an allosteric transition in the protein. To check that activity was not affected by loss of the positive charge of protein amino groups, the enzyme was incubated with fatty acid anhydrides, formaldehyde being unsuitable because of its release from proteins on dilution. None of the acids derived from the anhydrides activated the enzyme in this assay at 1 mM concentration and any possible effect was greatly reduced by the dilution. The effective half-life for hydrolysis of the anhydrides varied from 1–2 min for acetic and butyric anhydrides to approx. 20 min for decanoic anhydride; hence in the latter case the pro-

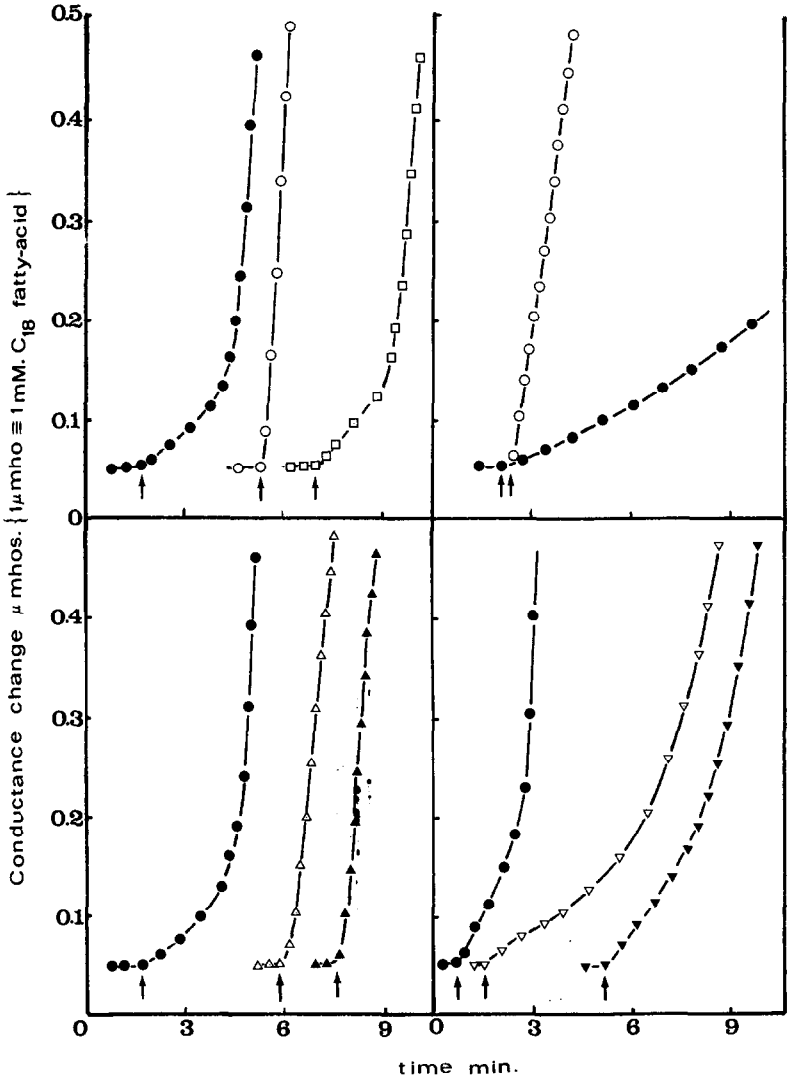

Fig. 1. The hydrolysis of 1.33 mM purified egg lecithin by 1 μg of purified Bee venom phospholipase A_2 at 37°C. a) Control (●–●–●), with 0.067 mM oleic acid (○–○–○) and with 0.044 mM lysolecithin (□–□–□). b(After incubation with 0.25% glutaraldehyde for 25 min (●–●–●) also in the presence of 0.024 mM oleic acid (○–○–○). c) After incubation with 3.2 mM decanoic anhydride and 0.38 mM oleic acid. Control (●–●–●), at 30 min (△–△–△) and at 60 min after extraction of anhydride with petroleum ether (▲–▲–▲). d) After incubation with 0.46 mM butyric anhydride and 0.08 mM oleic acid, assayed with 2 μg of enzyme. Control (●–●–●) at 2 min (▽–▽–▽) and at 88 min (▼–▼–▼).

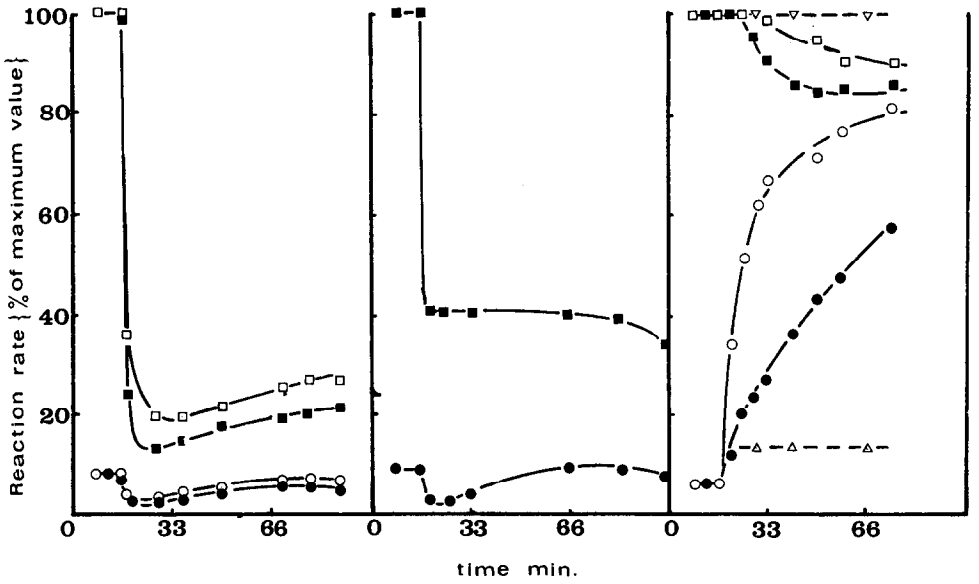

Fig. 2. The initial ○ ● △ and maximum □ ■ ▽ rates of hydrolysis of egg lecithin by phospholipase A_2 pre-incubated with aliphatic anhydrides. Assay and incubation conditions were as in fig. 1. a) Enzyme treated with 0.46 mM butyric anhydride in the absence ● ■ and presence ○ □ of 0.08 mM oleic acid. b) Treatment with 1.66 mM hexanoic anhydride. (The presence of oleic acid had no significant effect) c) Treatment with 3.2 mM decanoic anhydride, without ● ■ and with ○ □ 0.038 mM oleic acid. Also anhydride extracted with petroleum ether after 30 sec incubation △ ▽.

tein could be acylated throughout the 60–90 min incubation period, but in the former acylation ceases within 10 min.

From the results, figs. 1c, 1d and 2, we postulate that the protein has two sites (A and B), fig. 3a which on acylation (A*,B*) activate or inhibit the enzyme (subscripts a and i respectively) or do not affect activity, but block further acylation (subscript o). Site A activates (A_a^*) when occupied by long-chain acyl groups ($R>C_6$) or by free fatty acid ($R>C_{12}$) and is blocked by short acyl groups (A_o^*); the derivative is stable and acylation is catalysed by free fatty acid activators, fig. 2c, suggesting the model, fig 3b for the interaction.

Site B is inhibitory and overrides site A when occupied by short-chain acyl groups ($R<C_6$), but the derivative is unstable, fig. 1d, shown by the recovery of activity and by repetition of the inhibition cycle.

Lower anhydrides attack both sites, forming: $A_o^*B_i^*$, A_o^*B and AB_i^* which become A_o^*B and AB after de-acylation to give complete recovery of the initial rate and partial recovery of activatability, figs. 1d and 2a. Higher anhydrides activate, fig. 2c, by acylating site A, but either do not attack site B, or the derivative is very unstable, or else the modification is without effect on activity.

The results of hexamoic anhydride treatment, fig. 2b, suggest that the hexanoyl residue is a very weak activator at site A. It would be of interest to compare degree of activation with acyl chain length but slow irreversible inactivation occurs with all anhydride treatments and adequate correction cannot yet be made. Slow release of acyl groups from site B suggests that this site might be closely involved in the catalytic reaction, however the acylated derivative cannot lie on the normal reaction pathway because the hexanoyl residue, derived from a substrate, is released from the enzyme very rapidly.

Whether or not a single fatty acid binding site is responsible for all of the activation phenomena remains undecided, because the most highly activated enzyme, prepared by acylation and removal of excess anhydride by petroleum ether still has residual activatability (approx. 20%), fig. 1c. The facilitation of acylation by free activator could be evidence for two closely interacting fatty acid binding sites. In addition fatty acids

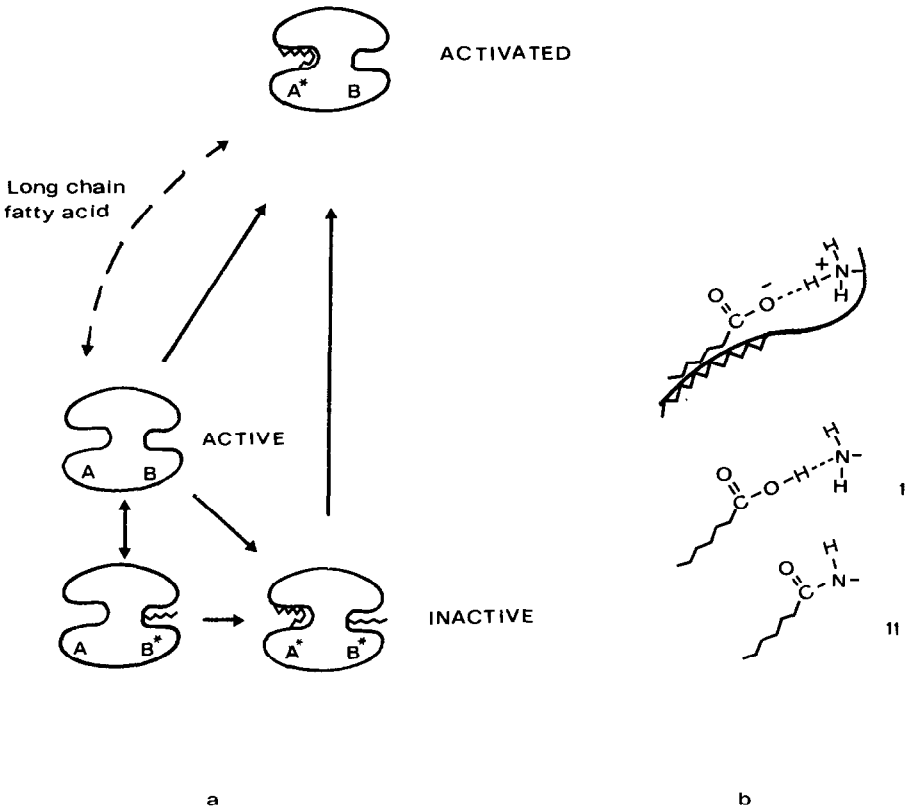

Fig. 3. a) Model for the effects of acylation on the activity of Bee venom phospolipase A_2. Site A is an allosteric activating site, operated either by freely interacting long-chain fatty acids or by acylation. Short acyl residues block the site and do not activate. Site B forms acyl derivatives which hydrolyse spontaneously causing temporary but complete inhibition. b) Partial model for the activating site. Fatty acids interact with a hydrophobic site to facilitate acylation of an amino group. (see fig. 2c) This suggests stabilisation of the uncharged structure 1, which closely resembles the acyl derivative 11.

give a small but significant reduction of early inhibition by lower anhydrides which could mean that they interact weakly at the inhibition site.

The theories of lipase activation by substrate modification have tended to obscure the possibility of specific action at the enzyme, and it would be surprising if the properties described above were unique to this enzyme.

Acknowledgement

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