REACTIVATION OF C_{55}-ISOPRENOID ALCOHOL PHOSPHOKINASE APOPROTEIN BY SYNTHETIC LECITHINS

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1. Introduction

The butanol-soluble enzyme C_{55}-isoprenoid alcohol phosphokinase catalyzes the ATP-dependent formation of C_{55}-isoprenoid alcohol monophosphate. This compound functions as a carrier molecule in the synthesis of several bacterial polysaccharides [1]. The enzyme requires a lipid cofactor for activity. A variety of lipids is able to satisfy this requirement which was shown to be non-electrostatic in nature. The important structural element appeared to be some physical parameter of the lipid [2].

With fatty acid auxotrophs of *E. coli* it has been demonstrated that a liquid-like state of the lipid phase is required for growth and for proper function of several membrane processes [3, 4]. Breaks in Arrhenius plots corresponded to phase transitions in monolayers of the isolated membrane lipids from a liquid-expanded to a condensed form [3].

Similarly the fluidity of the fatty acid chains of a lecithin cofactor might be important for the reactivation of IPA*. Characteristic phase transitions ("melting points") are known for lecithins in excess water. These occur at 23°, 0° and −22° for di-myristoyl-, di-lauroyl-, and di-oleyl-lecithin, respectively; [5]. The temperature dependence of the reactivation of IPA by these lecithins was investigated. Distinct breaks in Arrhenius plots were observed near 27°. However, these changes in activation energy do not appear to be due to the phase transition of a bulk lipid phase but rather to an unrelated reversible structural transition in a mixed micellar system that includes IPA.

* Abbreviations:
IPA: C_{55}-isoprenoid alcohol phosphokinase apoprotein;
PC: phosphatidylcholine.

2. Experimental

C_{55}-isoprenoid alcohol phosphokinase apoprotein was purified 1000-fold from membranes of *Staphylococcus aureus*, through step 6 (chromatography on DEAE-cellulose) of the purification procedure [6]. The solution used contained 20 μg/ml protein in methanol/n-butanol, 2:3, 0.6 M in ammonium acetate (Lowry-test). The average molecular weight of polypeptide chains present was about 10^4 [6]. ATP-γ-^{32}P was purchased from the Radiochemical Centre, Amersham. Ficaprenol was isolated from *Ficus elasticas* [7]. The synthetic lecithins were purchased from Supelco, Bellefonte, Pennsylvania. They were homogeneous on thin layer chromatography. Triton X-100 (average M.W. 628) was from Serva, Heidelberg.

3. Results

3.1. *Conditions required for reactivation of IPA*

By variation of the amount of lecithin included into the assay mixture an optimum concentration of about 2.5 mM was found for the 3 lecithins used (fig. 1).

With concentrations of di-palmitoyl-PC or di-stearoyl-PC higher than 0.2 mM a virtually complete inhibition of the enzyme reaction was obtained. This was at least partly due to traces of butanol which could not be removed in vacuo from these lipids by the normal assay procedure. Below 0.2 mM di-palmitoyl-L-lecithin and -D,L-lecithin as well as the di-hexadecyl-ether of L-lecithin (Calbiochem) showed about the same extent of reactivation. The 1-octadec-9-enyl,

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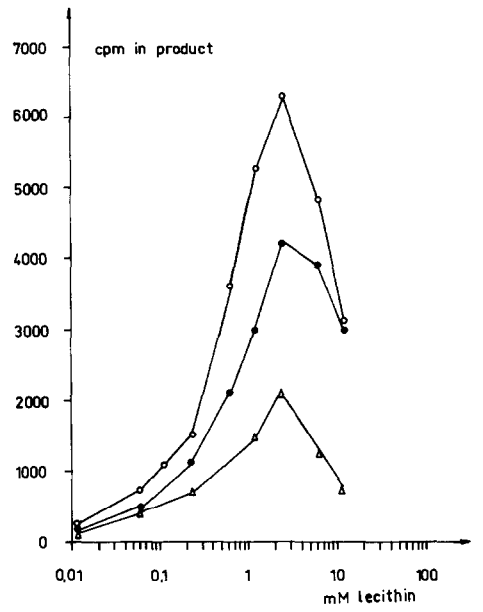

Fig. 1. Amount of lecithin required for reactivation of IPA. Di-oleyl-Lα-lecithin (○—○—○), di-myristoyl-Lα-lecithin (●—●—●) and di-lauroyl-Lα-lecithin (△—△—△) were employed in the following assay procedure: 1 μl of 16 mM ficaprenol and an aliquot of a lecithin solution in chloroform/methanol 2:1 were first dried in vacuo. 40 μl of enzyme was added and solvent was again removed in vacuo below 25°. The residue was suspended with a capillary tube in 25 μl buffer: 50 mM Tris-HCl, 10 mM $MgCl_2$, 4.8 mM Triton X-100, 10% dimethylsulfoxide, pH 8.5. The suspension was held at the temperature of the test (21°) for 2 min. The reaction was started by the addition of 1 μl of 25 mM ATP-γ-^{32}P (130,000 cpm). After 10 min the reaction was stopped by the addition of 25 μl tetrahydrofuran. The solution was applied to Whatman 3 MM paper and developed in isobutyric acid/1 M ammonia 5:3, by vol. Radioactivity associated with the front zone of the chromatogram containing ficaprenol-phosphate-^{32}P was determined in a liquid scintillation counter. The adjacent zone of the chromatogram was used to obtain a blank value

2-hexadecyl-ether of D,L-lecithin (Calbiochem) activated only slightly less than di-oleyl-Lα-lecithin over the entire concentration range tested. Thus the enzyme does not appear to be specific for the stereochemistry of the lecithins or for the ester linkages.

The optimum 2.5 mM concentration of lecithin represents a particularly high, about 800-fold molar excess relative to the number of polypeptide chains present, indicating a non-stoichiometric interaction of protein and lipid. From table 1 it appears that a 4.8 mM concentration of Triton X-100 or 20% dimethylsulfoxide cannot effectively substitute for the presence of di-oleyl-PC. The nearly absolute detergent requirement of the enzyme purified from the membrane of *S. aureus* as a lipoprotein [6] is not observed.

3.2. *Temperature dependence of the reactivation of IPA*

Arrhenius plots for the reactivation of IPA by three synthetic Lα-lecithins were determined at the optimum concentration of 2.5 mM (fig. 2).

In the presence of Triton X-100 and dimethylsulfoxide as well as with Tris buffer alone distinct

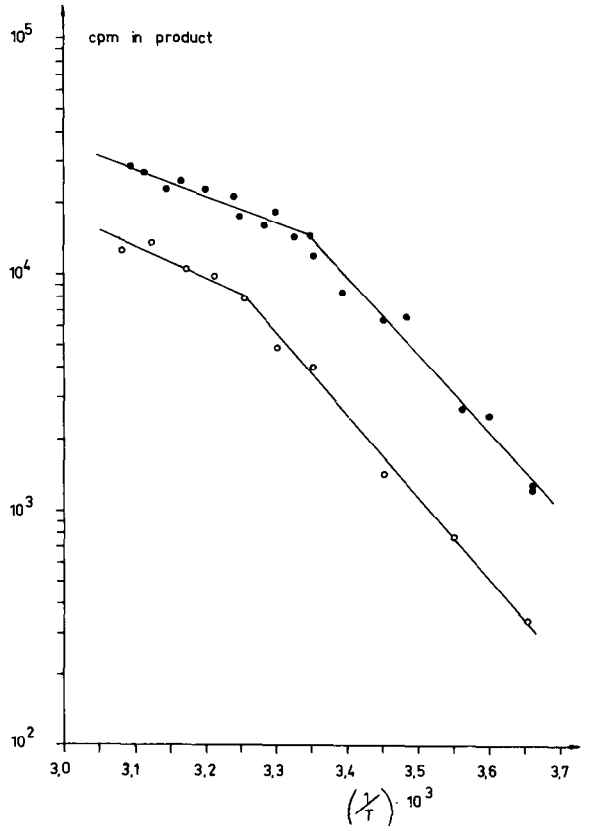

Fig. 2a. Temperature dependence of the reactivation of IPA by di-oleyl-Lα-lecithin (a), di-myristoyl-Lα-lecithin (b) and di-lauroyl-Lα-lecithin (c). 2.5 mM concentrations of the lecithins were used in the assay procedure described in the legend of fig. 1. The buffers employed in separate experiments were: 50 mM Tris-HCl, 10 mM $MgCl_2$, pH 8.5 (○—○—○); and 50 mM Tris-HCl, 10 mM $MgCl_2$, 4.8 mM Triton X-100, 10% dimethylsulfoxide, pH 8.5 (●—●—●). The amount of ficaprenol-^{32}P (cpm) formed is plotted against the reciprocal of the temperature of the assay (°K) on a semi-logarithmic scale.

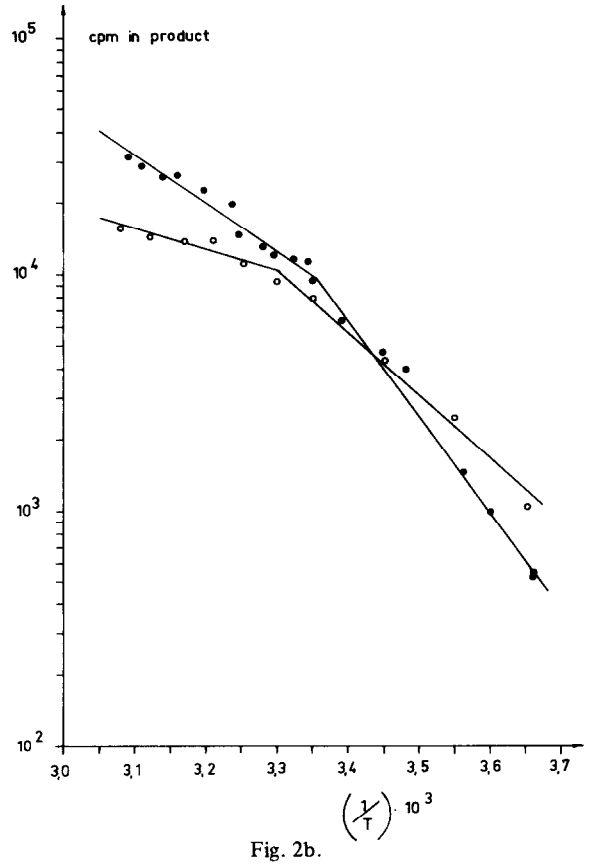

Fig. 2b.

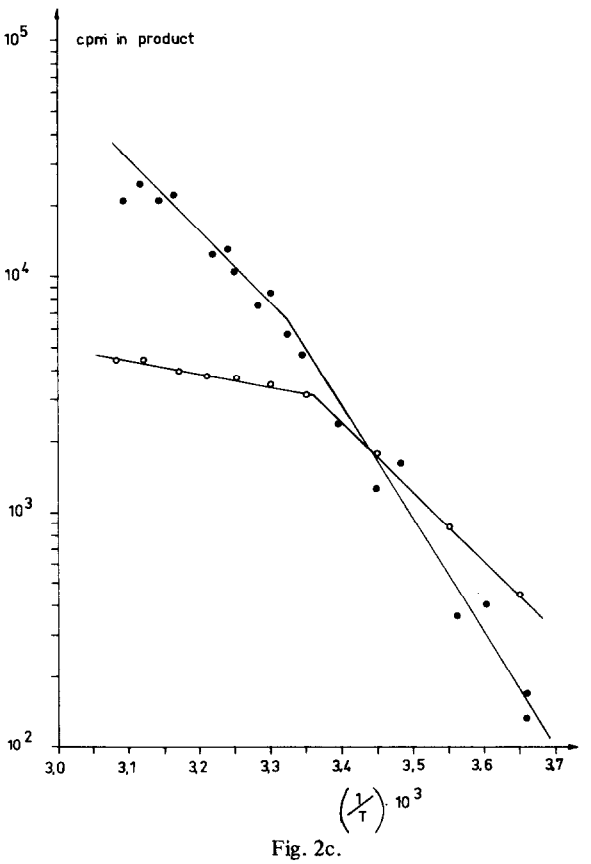

Fig. 2c.

Table 1
Buffer components required for reactivation of IPA.

	No lecithin present		Di-oleyl-lecithin present	
Buffer employed	Test at 24°	at 42°	Test at 24°	at 42°
Buffer A: 50 mM Tris-HCl, 10 mM $MgCl_2$, pH 8.5	20	100	4,700	14,200
Buffer A + 20% dimethyl-sulfoxide	15	120	3,800	11,000
Buffer A + 4.8 mM Triton X-100	300	1,200	12,000	22,600
Buffer A + 20% dimethyl-sulfoxide + 4.8 mM Triton X-100	600	3,800	12,600	23,800

Assays were carried out as described in the legend of fig. 1 either in the absence or in the presence of 2.5 mM di-oleyl-Lα lecithin. The buffers employed and the temperatures of the incubations are indicated in the table. Cpm in product are shown. The total amount of radioactivity used was 140,000 cpm.

Table 2
Arrhenius activation energies (kcal/mole) and transition temperatures.

	Transition temp. (°C)	Arrhenius activation energies lower part	upper part	diff.	Transition temp. (°C)	Arrhenius activation energies lower part	upper part	diff.
Di-lauroyl-PC	24.3°	13.6	2.7	10.9	28.0°	21.8	13.9	7.9
Di-myristoyl-PC	29.5°	12.5	4.2	8.3	24.7°	18.8	9.1	9.7
Di-oleyl-PC	34.4°	15.8	6.2	9.6	26.5°	14.8	5.2	9.6

The curves shown in fig. 2 were used for the calculations.

Table 3
Stability of the enzyme.

Lecithin used:	Di-oleyl-PC	Di-myristoyl-PC	Di-lauroyl-PC
Set I	5,900	5,400	2,300
Set II	19,200	20,200	19,500
Set III	6,200 (105)	5,000 (93)	1,800 (78)
Set IV	5,900 (100)	4,500 (84)	1,550 (68)
Set V	7,300 (124)	4,400 (82)	1,900 (83)
Set VI	18,200 (95)	16,700 (83)	13,500 (69)

The indicated lecithins (2.5 mM) were used in the standard assay (see legend of fig. 1). Sets I and II were incubated at 20° and 45.5°, respectively, in the usual way. Total cpm employed were 125,000. The values obtained were used as reference values (100%) for comparison with incubations III–VI. Set III: The assay mixture was kept at 20° for 20 min prior to the addition of ATP and incubation at 20°. Set IV: The assay mixture was kept at 45.5° for 20 min prior to the addition of ATP and incubation at 20°. Set V: The assay mixture was kept at the following temperatures prior to the addition of ATP and incubation at 20°: 10 min/45.5°, 10 min/20°, 10 min/45.5°. Set VI: The assay mixture was kept at the following temperatures prior to the addition of ATP and incubation at 45.5°: 10 min/45.5°, 10 min/20°, 10 min/45.5°, 10 min/20°. Yields in % relative to the reference values are indicated in brackets.

breaks are visible near a temperature of 27°. The exact transition temperatures were dependent on the composition of the incubation mixture and are shown in table 2. The Arrhenius energies of activation were calculated from the slopes of the curves obtained and are also given in table 2. The differences between the individual activation energies are outside of experimental error.

An attempt was made to rule out the occurrence of some irreversible process (table 3). A significant decrease of enzyme activity after treatment at higher temperature was noted only with di-lauroyl-PC.

4. Discussion

Clearly the sudden changes in activation energy observed cannot be correlated with the phase transitions at 23°, 0° and −22° [5] known for the lecithins alone in excess water. In fact, it is doubtful whether these phase transitions will occur in the presence of 40 mole-% of ficaprenol or of a 2-fold molar excess of Triton X-100. The phase transition of di-palmitoyl-PC is abolished by about 32 mole-% of cholesterol [5].

An alternative explanation would be a conformational change of the enzyme protein near 27°, as has been described for D-amino acid oxidase [8].

However, the transition temperatures observed as well as the activation energies below and above the transition temperatures differ significantly with the fatty acid substitution of the three lecithins used (table 2). Also the effect of Triton X-100 is different with the lecithins employed.

It can be assumed that the detergent Triton X-100,

the lipid substrate ficaprenol and the lecithin "cofactor" will form mixed micelles† rather than occur separately in aqueous dispersion [9]. The enzyme apoprotein probably is also at least partially incorporated into the hydrocarbon interior of the micellar system. IPA is found in the butanol layer when equilibrated in a butanol/water 2-phase system [2]. Also it is clear that ionic interactions do not contribute to the reactivation of IPA by lipophilic activators [2]. The mixed micellar system must allow the approach of ATP to a binding site of IPA for the enzyme reaction to proceed. In summary the enzyme reaction is thought to occur at the interphase of a mixed micellar system and water. The breaks observed in Arrhenius plots (fig. 2) could then correspond to a reversible conformational change of IPA and its micellar environment.

Growth and several membrane processes of intact bacteria show sudden changes in activation energies near the temperatures where phase transitions of the isolated membrane lipids are observed [3, 4]. This phenomenon is probably related to the change in permeability accompanying lipid phase transitions. No breaks in Arrheneius plots are observed when permeability barriers are reduced by disrupting the bacterial cells [10]. Triton X-100 effectively disrupts lipid structures [11] but did not abolish the breaks observed in the reactivation of IPA (see fig. 2).

Very little is known at present about enzyme reactions in micellar systems or about conformational properties of IPA. The identification of the structural transition discussed therefore has to await further study.

† The general term micelle is used here without referring to any of the various polymorphic structures that are known for amphipathic molecules in excess water [9].

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