Biochimica et Biophysica Acta, 635 (1981) 348—358

BBA 48035

TRANSMEMBRANE DISTRIBUTION OF PHOSPHOLIPIDS AND THEIR INVOLVEMENT IN ELECTRON TRANSPORT, AS REVEALED BY PHOSPHOLIPASE A_2 TREATMENT OF SPINACH THYLAKOIDS

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(Received October 28th, 1980)

Key words: Phospholipid distribution; Electron transport; Phospholipase A_2; Chloroplast; (Thylakoid membranes)

Summary

Thylakoid membranes were treated with either pancreatic or snake venom phospholipase A_2, and the residual phospholipid content of these membranes was determined and compared to the rates of Photosystem II and/or Photosystem I electron transports. The hydrolysis curves of both phosphatidylglycerol and phosphatidylcholine displayed a first, rapid phase which was almost temperature-insensitive, followed by a second, slower phase which depended strongly on the temperature. When pancreatic phospholipase A_2 had access either to the outer face or to both faces of the thylakoid membrane, either only part of or all the phospholipids, respectively, could be hydrolysed. These results were interpreted as indicating an asymmetric distribution of phospholipids across the thylakoid membrane, phosphatidylglycerol and phosphatidylcholine being preferentially located in the outer and the inner layer, respectively. When acting on uncoupled thylakoid membranes, phospholipase A_2 exerted an inhibitory effect on Photosystem II activity and a stimulatory effect on Photosystem I activity. The involvement of phosphatidylcholine and of phosphatidylglycerol in electron transport activities of Photosystem II and of Photosystem I are discussed with special reference to the role of the external and internal pools of these phospholipids.

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* This work is part of a doctoral program which is being carried out by A.R. in the Laboratoire de Physiologie Végétale et Biochimie, Université de Neuchâtel, Neuchâtel, Switzerland.

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Abbreviations: DCMU, 3-(3,4-dichlorophenyl)-1,1-dimethylurea; DCIP, 2,6-dichlorophenolindophenol; Hepes, *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid; Mops, 3-(*N*-morpholino)propanesulfonic acid; Tricine, *N*-tris(hydroxymethyl)methylglycine.

Introduction

The relationship between the functional asymmetry of the thylakoid membrane and its structural sidedness, thoroughly discussed in 1974 by Trebst [1], has gained continuous support during the past six years. So far, this relationship is mainly concerned with the proteins involved in electron transport and proton movements. However, our knowledge of the distribution of lipids in thylakoid membranes is rather scarce [2—4]. The only major contribution to the problem of the localization of lipids in chloroplast membrane fractions was made by the group of Radunz (Ref. 5 and references therein), who used immunological techniques. However, this approach did not provide a quantitative estimation of the transmembrane distribution of lipids, although an asymmetric picture of the thylakoid membrane emerged from these studies.

Lipolytic treatments of biological membranes were designed for a double aim. Firstly, they have been widely used with bacterial and animal membranes in an attempt to establish the quantitative distribution of lipids with respect to a possible asymmetry of these components across the membrane. Advantages and limitations of this approach have been critically reviewed recently by Op den Kamp [6]. Secondly, lipolytic treatments were often used to assess the direct involvement of lipids in the functionality of various membranes. This latter approach was also applied to thylakoid membranes [7—20]; however, the results were often ambiguous [8—20]. In our view, there are at least two reasons for this lack of clear-cut results. Most authors did not recognize clearly that a lipolytic enzyme destroys almost inevitably lipids which do not support directly the measured function. In addition, the fact that biological membranes are generally described in terms of a lipid bilayer, the outer and inner monolayers of which being mutually independent up to a certain degree [21], was neglected in these studies [8—20]. We believe that the knowledge of the distribution of lipids across the thylakoid membrane is a prerequisite to any attempt to understand whether and, if so, how the lipids are involved in the functions of this membrane.

In this investigation, we present the thylakoid transmembrane distribution of phosphatidylcholine and of phosphatidylglycerol as revealed by pancreatic phospholipase A_2 treatment. In addition, we report on the role of external and internal pools of these phospholipids in sustaining electron transport activities in spinach thylakoid membranes. A preliminary report of these findings has been published [22].

Materials and Methods

Enzymes

Phospholipases A_2 (EC 3.1.1.4) from pig pancreas and from *Vipera russelli* venom, purchased from Sigma Chemical Co., were used without further purification.

Preparation of thylakoid membranes

Spinacia oleracea (var. Nobel) was grown in a growth chamber and the leaves

were harvested after 14 weeks of hydroponic culture [23]. Intact chloroplasts were first prepared according to Kalberer et al. [24] in a medium containing 25 mM Mops (pH 7.6) and 350 mM sucrose, spun down at 1935 × *g* for 30 s and then osmotically disrupted in the above medium diluted 10-fold. Centrifugation at 17 300 × *g* yielded a pellet which was resuspended in a medium containing 25 mM Mops (pH 7.6) and 175 mM NaCl. The chlorophyll concentration was determined spectrophotometrically [25] and adjusted to 2 mg/ml; this suspension was used without delay for the enzymatic treatments.

Enzymatic treatments

The incubation of thylakoid membranes (0.5 mg chlorophyll/ml) with enzymes was carried out in darkness in 50 mM Tricine (pH 8), 80 mM NaCl, 1 mM $CaCl_2$ and phospholipase A_2 either from pig pancreas (not less than 1.6 Sigma unit/mg chlorophyll) or from *Vipera russelli* venom (not less than 0.11 Sigma unit/mg chlorophyll). Except where indicated, the incubations were performed at 20°C. Aliquots of the above incubation media were taken at various times (including a zero time control) for the determination of electron transport activities and for lipid analysis.

Determination of electron flow activities

Photosystem II activity was measured by the photoreduction of DCIP at 590 nm in a glass cuvette (0.2 cm optical path); the reaction mixture contained 50 mM Hepes (pH 7.6), 35 mM NaCl, 0.2 mM DCIP, 0.005 mM 2,5-dibromo-3-methyl-6-isopropyl-*p*-benzoquinone and thylakoid membranes (40 μg chlorophyll/ml). When used instead of water as an electron donor, 1,5-diphenylcarbazide was at 0.5 mM in the reaction mixture.

Photosystem I activity was estimated by the photoreduction of $NADP^+$ at 340 nm; the reaction mixture contained 50 mM Tricine (pH 8.4), 35 mM NaCl, 4 mM sodium ascorbate, 0.3 mM DCIP, 0.01 mM DCMU, 2 mM $NADP^+$, 6 μM ferredoxin and thylakoid membranes (100 μg chlorophyll/ml).

Photosystem II + I activity was measured as a $H_2O/NADP^+$ electron flow. The reaction mixture was the same as for Photosystem I, except that ascorbate, DCIP and DCMU were omitted. When the H_2O/methylviologen electron flow activity was followed with a Clark oxygen electrode, the reaction mixture contained 50 mM Tricine (pH 8.4), 35 mM NaCl, 2 mM NaN_3, 0.15 mM methylviologen and thylakoid membranes (20 μg chlorophyll/ml).

The intensity of actinic light after filtration through a Calflex and a DT-Red filter (both from Balzers) was 250 mW · cm^{-2} at the level of the cuvettes.

Lipid analysis

The changes in the phospholipid content of thylakoid membranes treated with phospholipases A_2 were followed by the method of Rawyler and Siegenthaler [26]. This method is based on the combination of thin-layer chromatography and scanning of photographic negatives of the charred plates. The results are expressed as percentages of the initial (control) values. The amounts of phosphatidylglycerol and phosphatidylcholine in freshly isolated thylakoid membranes were 0.195 and 0.150 μmol/μmol chlorophyll, respectively [26].

Results and Discussion

When thylakoid membranes were treated with pancreatic phospholipase A_2, the hydrolysis of phosphatidylglycerol and phosphatidylcholine displayed two phases (Fig. 1). The first was almost temperature-independent whilst the second was strongly dependent on this factor. The extent and rate of the first, fast hydrolytic step were greater for phosphatidylglycerol than for phosphatidylcholine. The difference in rates reflects, at least in part, the preference of the pancreatic phospholipase A_2 for anionic phospholipids whilst the difference in the extents of the first hydrolytic step suggests that a greater amount of phosphatidylglycerol than of phosphatidylcholine is easily accessible to the enzyme. The rates of the second phase were much slower; however, above 15°C the hydrolysis of both lipids was eventually complete. These results may indicate an asymmetric distribution of the phospholipids in the thylakoid membrane.

In order for this to be true, at least three criteria should be satisfied in control experiments [27]. Firstly, no lysis of the thylakoids should occur when the enzyme attacks the membrane from the outside. This was indirectly established by the fact that a proton gradient was still maintained across the membrane when the first hydrolytic step was achieved (data not shown). Secondly, all lipids should be hydrolyzed when the enzyme attacks the membrane from both sides. Fig. 2 shows that when phospholipase A_2 attacks the membrane from the outside only (e.g. in normal thylakoids), the hydrolysis of both phospholipids at 0°C was only partial. At this temperature the transbilayer movement mechanism is negligible. On the other hand, when phospholipase A_2 had access to both sides, e.g. in disrupted thylakoids (Fig. 2), the hydrolysis of phosphatidylglycerol and phosphatidylcholine was almost complete. This indicates that some of the phospholipids are located in the inner layer of the thyla-

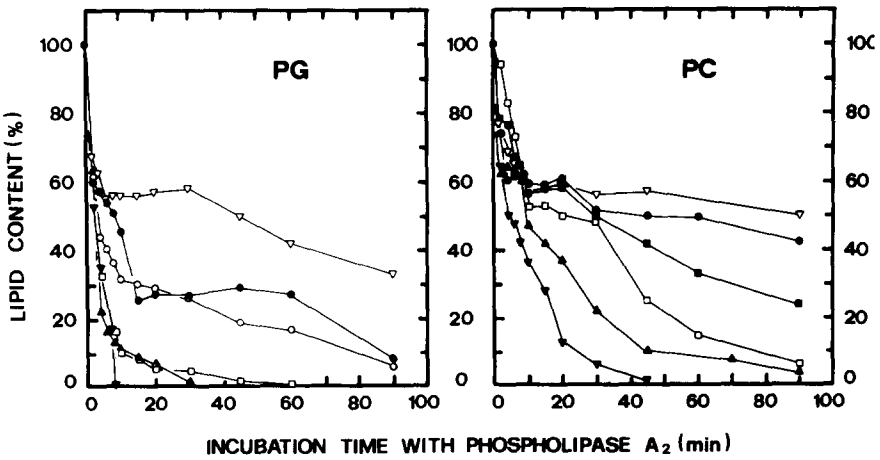

Fig. 1. Time course of phospholipid hydrolysis at various temperatures in thylakoid membranes treated with pancreatic phospholipase A_2. ▽———▽, 0°C; ●———●, 5°C; ○———○, 10°C; ■———■, 15°C; □———□, 20°C; ▲———▲, 25°C; ▼———▼, 35°C. PG, phosphatidylglycerol; PC, phosphatidylcholine.

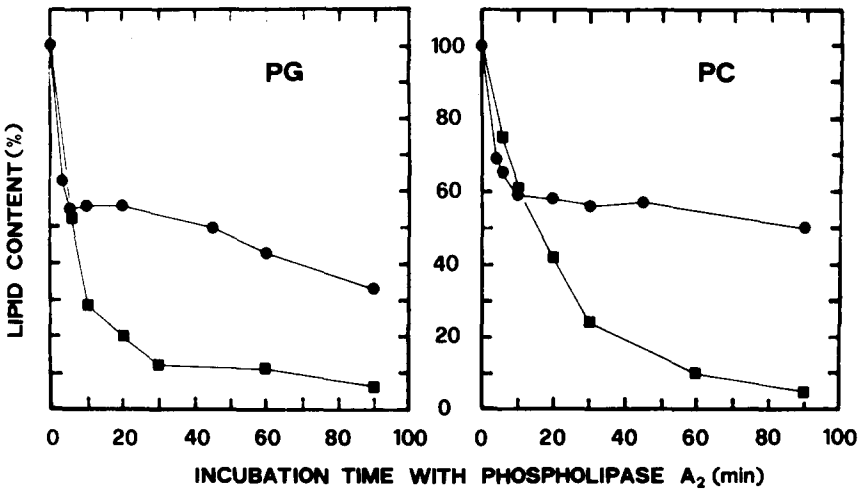

Fig. 2. Time course of phospholipid hydrolysis at 0°C in normal (●——●) and disrupted (■——■) thylakoid membranes treated with pancreatic phospholipase A_2. Disrupted thylakoid membranes were obtained by sonication of normal membranes for 3 min in an incubation mixture containing phospholipase A_2, but no NaCl and no $CaCl_2$; vesicles containing the trapped enzyme were thus formed and Ca^{2+} was added to initiate the hydrolytic reaction. PG, phosphatidylglycerol; PC, phosphatidylcholine.

koid membrane. Thirdly, an unequivocal localization of the lipids in the membrane can only be obtained if the lipolytic treatment does not modify the actual distribution of these lipids. This implies that no transbilayer movement should occur during the hydrolytic process. Although this was the case at low temperatures, the intensity of this phenomenon increased when the temperature was raised (see second phases in Figs. 1 and 2). Consequently, the best estimation of the actual distribution of phospholipids in the membrane is given by computing the fast-reacting pools of phosphatidylglycerol and phosphatidyl-

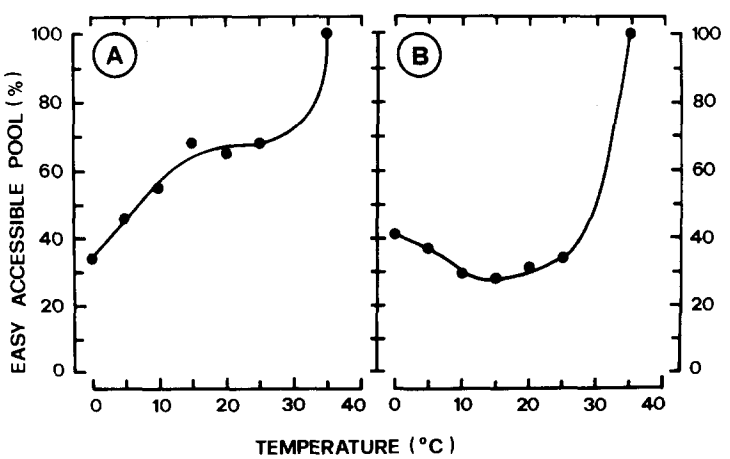

Fig. 3. Effect of temperature on the easy accessible pools of phosphatidylglycerol (A) and phosphatidylcholine (B) in thylakoid membranes treated with pancreatic phospholipase A_2. The estimation of these pools was made from the data of Fig. 1 by the logarithmic treatment of Demant et al. [28].

choline as shown in Fig. 3. The easily accessible pool of phosphatidylglycerol, which was 35% at 0°C, increased with temperature to reach a plateau between 15 and 25°C. On the other hand, the corresponding pool of phosphatidylcholine remained essentially constant within 30—40% of the total phosphatidylcholine between 0 and 25°C. Above 25°C, the accessibility of both lipids became total. We conclude that around 70% of phosphatidylglycerol and 35% of phosphatidylcholine could be localized in the outer layer of the thylakoid membrane.

Previous reports have shown that phospholipase A_2 treatment of spinach [19] and pea [20] thylakoid membranes resulted in a rapid hydrolysis of about 80% of the total phosphatidylglycerol. Our data on phosphatidylglycerol are in good agreement with these results, although these earlier works were not aimed at the study of the phospholipid transmembrane distribution. Moreover, our results give a quantitative support to the qualitative estimation of the distribution of phosphatidylglycerol in *Antirrhinum* thylakoid membranes [5]. In contrast, the distribution of phosphatidylcholine presented here is just the opposite of that suggested by Radunz [5].

The effects of pancreatic phospholipase A_2 at 20°C on different electron flow activities of thylakoid membranes are shown in Fig. 4. The activities involving Photosystem II + I (Fig. 4 A and B) decreased rapidly and were completely

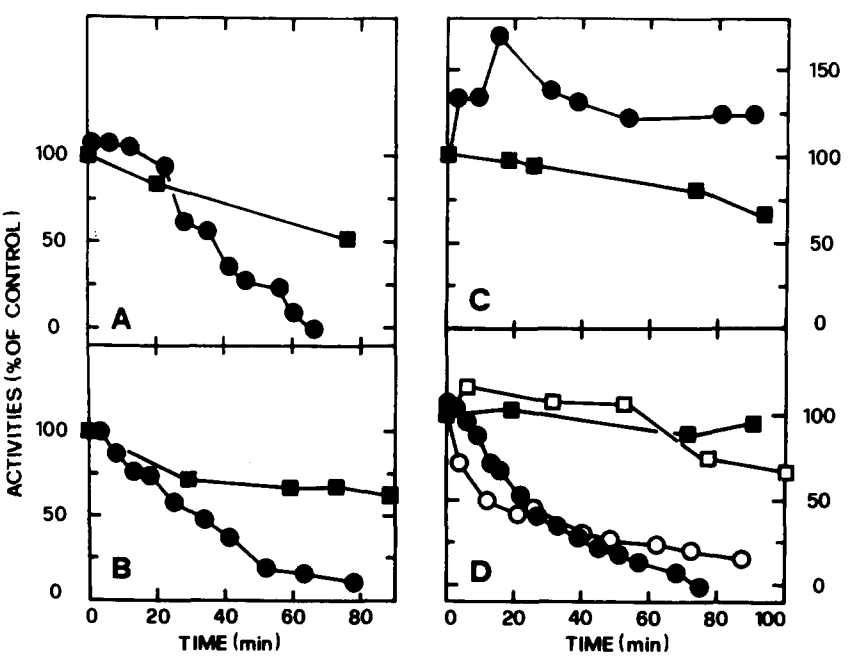

Fig. 4. Action of pancreatic phospholipase A_2 on thylakoid membranes followed by the time-dependent variations of electron transport activities measured from H_2O to $NADP^+$ (A) or to methylviologen (B) (Photosystem II + I), from reduced DCIP to $NADP^+$ (C) (Photosystem I) and from H_2O (closed symbols) or 1,5-diphenylcarbazide (open symbols) to DCIP (D) (Photosystem II). □ and ■, control membranes; ○ and ●, treated membranes. The 100% values were 140 and 70 μmol $NADPH_2 \cdot mg^{-1}$ chlorophyll $\cdot h^{-1}$ in A and C, respectively; 80 μmol O_2 consumed $\cdot mg^{-1}$ chlorophyll $\cdot h^{-1}$ in B and 130 μmol $DCIPH_2 \cdot mg^{-1}$ chlorophyll $\cdot h^{-1}$ in D.

inhibited after 60 to 80 min. In contrast, phospholipase A_2 brought about a stimulation of Photosystem I activity which remained higher than the control (Fig. 4C). A greater activation was found when methylviologen was used as the electron acceptor (data not shown). This stimulation was only partially due to uncoupling [9] since the long-term activation was also observed in uncoupled thylakoid membranes. The inhibition of electron flow through both photosystems (Fig. 4 A and B) could be ascribed to the decrease in Photosystem II activity as shown in Fig. 4D. Comparing the 1,5-diphenylcarbazide- and H_2O-supported Photosystem II activities, it seems unlikely that the water-splitting system is inactivated by the phospholipase A_2 treatment. Thus, these results suggest the existence of at least two action sites of phospholipase A_2. The first, leading to an inhibitory effect, is located between the electron entry point from 1,5-diphenylcarbazide and the plastoquinone pool. The second site, leading to a stimulatory effect, is located between the electron entry point from $DCIPH_2$ and the primary acceptor of Photosystem I.

These results are in partial agreement with those of Hirayama and Matsui [17] but disagree strongly with more recent works by Hirayama and Nomotobori [19] and Duval et al. [20]. Indeed, our phospholipase A_2 treatments always led to an inhibition of Photosystem II electron transport activity which was not found by these authors [19,20]. This discrepancy can be explained, at least in part, by the fact that these two groups did not add 2,5-dibromo-3-methyl-6-isopropyl-*p*-benzoquinone to their H_2O/DCIP reaction mixture and thus did not measure a true Photosystem II-mediated electron flow, but a preferential DCIP reduction through Photosystem I [29,30]. Moreover, it should be emphasized that the hydrolysis of the lipid moiety of membranes, treated either with the lipolytic acyl hydrolase from potato [7] or with the pancreatic and snake venom phospholipase A_2 (see Figs. 1, 4 and 5), begins before any

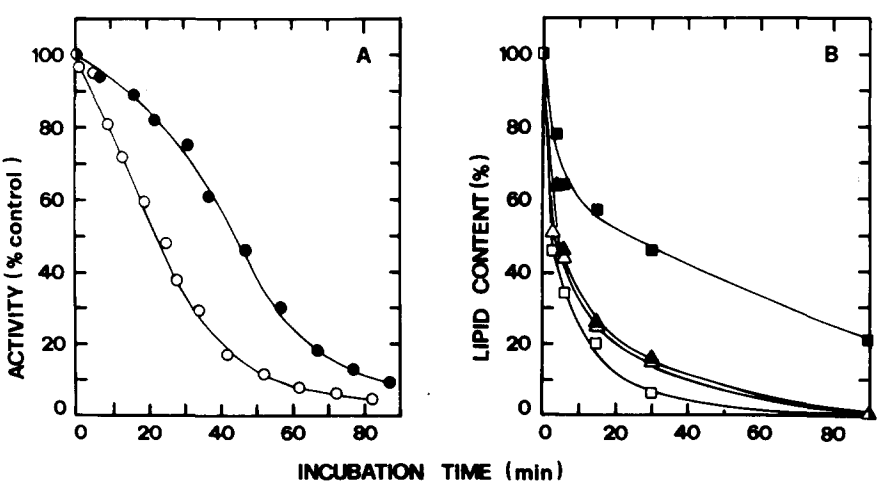

Fig. 5. Comparison of the action of pancreatic and *Vipera russelli* venom phospholipase A_2 on Photosystem II electron flow activity (A) and on the hydrolysis of phospholipids (B) as a function of incubation time. Closed symbols: pancreatic phospholipase A_2; open symbols: snake venom phospholipase A_2. A, Photosystem II electron flow H_2O/DCIP; B, phospholipid content; ▲ and △, phosphatidylglycerol; ■ and □, phosphatidylcholine.

appreciable change in electron flow activities can be detected. Hirayama and Nomotobori [19] and Duval et al. [20] did not present the time-dependent changes in electron flow activities of their phospholipase A_2-treated thylakoid membranes. Thus, the weak inhibition of Photosystem II electron flow activity observed by these authors could be explained by the limited time of incubation of their membranes in the presence of phospholipase A_2.

The shapes of the hydrolysis curve of phosphatidylcholine at 20°C (Fig. 1) and of the decay in Photosystem II activity (Fig. 4D) seem to indicate a possible participation of phosphatidylcholine in Photosystem II structures. The comparison between the action of phospholipase A_2 from pancreas and snake venom (which have inverse preference toward phosphatidylcholine and phosphatidylglycerol) supports the hypothesis that phosphatidylcholine is the main phospholipid involved in Photosystem II electron flow activities (Fig. 5). When the concentrations of both enzymes were chosen so as to obtain the same hydrolysis rate of phosphatidylglycerol, the extent and rate of the hydrolysis of phosphatidylcholine by the pancreatic phospholipase A_2 were smaller than that by the snake venom enzyme (Fig. 5B). This difference in the hydrolytic patterns of phosphatidylcholine can be related to the inhibition of Photosystem II electron flow activity which was smaller with pancreatic phospholipase A_2 than with the snake venom enzyme (Fig. 5A).

Again, this result is not in agreement with that of Duval et al. [20] who suggested that phosphatidylglycerol was mainly associated with Photosystem II structures. These authors have apparently neglected the fact that the phospholipase A_2 from *Vipera russelli* venom shows a preference for zwitterionic phospholipids such as phosphatidylcholine as compared to anionic phospholipids such as phosphatidylglycerol [31]. Consequently, they did not measure the changes in the phosphatidylcholine content of their phospholipase A_2-treated thylakoids, considering that the phosphatidylcholine level was constant. This was obviously not the case (Fig. 5B).

The involvement of phosphatidylcholine in Photosystem II-mediated activity is further substantiated by the comparison, at three different temperatures, of the action of pancreatic phospholipase A_2 on the phospholipid content and on Photosystem II electron transport activities in uncoupled thylakoid membranes. When the external pool of phosphatidylcholine was hydrolyzed (for instance up to 10 min of phospholipase A_2 treatment at 5°C and at 20°C, Fig. 6a and b) no change in Photosystem II activity was observed. However, the hydrolysis of the internal pool of phosphatidylcholine under the conditions where transbilayer movement occurs [28] (for instance at 35°C and after 10 min of phospholipase A_2 treatment at 20°C, Fig. 6 b and c) was directly related to the inhibition of Photosystem II activity. On the other hand, when no transbilayer movement occurred (for instance after 10 min of phospholipase A_2 treatment at 5°C) there was no appreciable inhibition of the Photosystem II activity. In addition, Fig. 6 shows that the hydrolysis of the external pool of phosphatidylglycerol was associated with an increase in Photosystem I activity, which was markedly stimulated when the internal pool of phosphatidylglycerol was further hydrolyzed, due to the transbilayer movement. It is noteworthy that the great stimulation of Photosystem I activity is not due to uncoupling. This unexpected observation suggests that phosphatidylglycerol plays

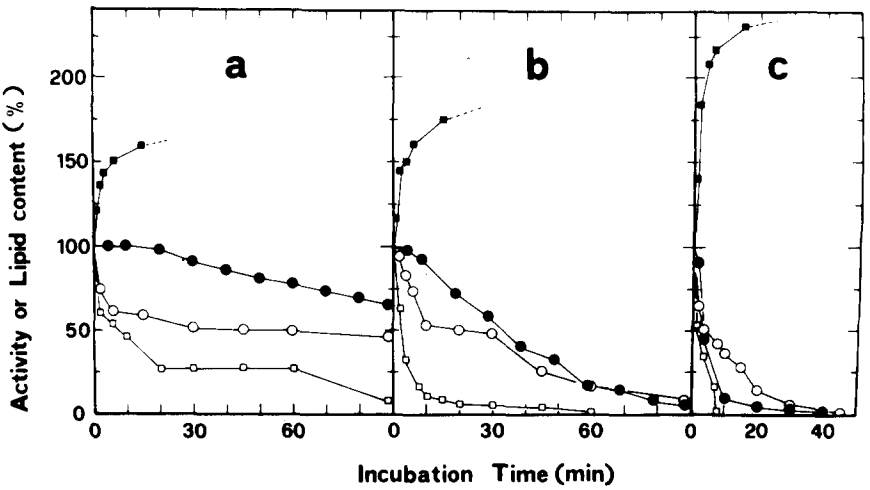

Fig. 6. Temperature dependence of the action of pancreatic phospholipase A_2 on the phospholipid content and on Photosystem II and Photosystem I electron transport activities of uncoupled thylakoid membranes. a, 5°C; b, 20°C; c, 35°C. ●——●, Photosystem II electron flow, H_2O/DCIP; ■——■, Photosystem I electron flow, $DCIPH_2/NADP^+$; ○——○, phosphatidylcholine; □——□, phosphatidylglycerol. Uncoupling and blocking of hydrolysis were achieved by addition of 2 mM EDTA in the reaction media.

a privileged role in the function of Photosystem I. These results point out the great importance of the internal pool of phosphatidylcholine to sustain the Photosystem II electron transport activity and the complex relationship between both external and internal pools of phosphatidylglycerol and the

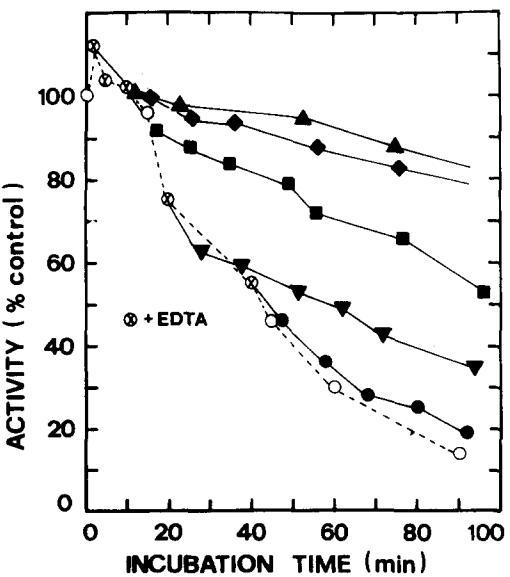

Fig. 7. Effect of various incubation times on the Photosystem II-catalysed photoreduction of DCIP by water in thylakoid membranes treated with pancreatic phospholipase A_2. Hydrolysis is blocked by addition of 2 mM EDTA (⊗) after 2 (▲), 5 (◆), 10 (■), 20 (▼) and 40 min (●) of treatment. Non-interrupted treatment with phospholipase A_2 (○,⊗). Incubations were made at 20°C.

Photosystem I electron flow activity.

Another approach to show the importance of the internal phosphatidylcholine was to control the degree of phospholipid depletion of thylakoid membranes and to observe the subsequent behavior of Photosystem II electron flow activity. Such an experiment is shown in Fig. 7 where EDTA was added in the incubation mixture to block phospholipase A_2 action by Ca^{2+} chelation. Short treatments (2 and 5 min) with phospholipase A_2, which destroyed only partially the external pool of phosphatidylcholine (Figs. 1 and 6 a and b) and which do not induce a sizable transbilayer movement, did not inhibit significantly Photosystem II activity (Fig. 7). In contrast, longer treatments with phospholipase A_2, which causes an increasing transbilayer movement and a progressive delocalization of internal phospholipids, did inactivate Photosystem II electron flow. However, the delocalization of internal phosphatidylcholine, but not its further destruction by phospholipase A_2, was in itself necessary and sufficient to cause the inhibition of Photosystem II activity (compare Figs. 6b and 7). It is noteworthy that the presence of lyso-compounds produced in situ did not appear to contribute significantly to the inactivation of electron flow. Indeed, even if all phosphatidylcholine is converted into its lyso-derivative in phospholipase A_2-treated thylakoid membranes, the molar ratio of lysophosphatidylcholine to chlorophyll (0.15) is far below that (0.70) required to obtain a detectable inhibition on the Photosystem II electron flow activity [32,33]. It must be emphasized that this threshold value of 0.70 was obtained with various amounts of lysophosphatidylcholine added to thylakoid membranes [32,33]; moreover, a given amount of lysophosphatidylcholine [34] or free fatty acids [35] was much more inhibitory when added exogenously than when the same amount was released in situ by lipolytic enzymes.

General conclusions

Treatments of thylakoid membranes by phospholipase A_2 permit us to draw the following conclusions:

1. Phospholipids are asymmetrically distributed in the thylakoid membrane. The outer layer is enriched in phosphatidylglycerol whereas the inner layer is enriched in phosphatidylcholine.

2. Phosphatidylcholine is the main phospholipid associated with the thylakoid structures responsible for Photosystem II electron flow. The presence of an intact pool of phosphatidylcholine in the inner layer of the membrane is absolutely required for maximal Photosystem II activity.

3. Phosphatidylglycerol appears to be preferentially associated with the thylakoid structures responsible for Photosystem I electron flow. Both outer and inner pools of phosphatidylglycerol seem to be involved in the modulation of this activity.

4. The role of a given lipid in a thylakoid membrane function is determined by its transmembrane distribution, its localization in the plane of the two leaflets of the membrane and by its chemical identity, but not necessarily by its relative abundance in the membrane.

The above results favor the idea that the well-known sidedness of the thyla-

koid membrane is supported in part by an asymmetric distribution of its phospholipids.

Acknowledgements

We thank Miss Marie-Claire Pfeiffer for her able technical assistance and the Swiss National Science Foundation for its support (Grant 3.119.77 and 3.661.80 to P.A.S.).

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