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Phospholipid Topography of the Photosynthetic Membrane of *Rhodopseudomonas sphaeroides*†

Khalid K. Al-Bayatti and Jon Y. Takemoto*

ABSTRACT: The topography of phospholipids in the photosynthetic membranes of *Rhodopseudomonas sphaeroides* was investigated by using purified chromatophores and spheroplast-derived vesicles (SDVs). Chromatophores are closed vesicles oriented inside out with respect to the cytoplasmic membrane (cytoplasmic side out) and obtained from French-pressed cell lysates. SDVs are oriented right side out (periplasmic side out) and are obtained after osmotic lysis of lysozyme-treated cells. Phosphatidylethanolamine (PE) comprised ~62% and phosphatidylglycerol (PG) comprised ~33% of the total phospholipid of both vesicle preparations. The relatively membrane impermeable reagent trinitrobenzenesulfonate (TNBS) at 3 mM concentration and 5 °C modified chromatophore and SDV PE with kinetics indicating the occurrence of fast- and slow-reacting pools of PE. The fast-reacting pools comprised 33% and 55% of the total PE of chromatophores and SDVs, respectively. The slow-reacting pools comprised 61% and 32% of the total PE of chromatophores and SDVs, respectively. Phospholipase A_2 treatment of chromatophores (1 unit/mg of vesicle protein) for 1 h at 37 °C resulted in hydrolysis of 73% and 77% of the total PG and PE, respectively. Similar enzyme treatment of SDVs resulted in 14% and 60% hydrolysis of the total PG and PE, respectively. Phospholipase A_2 treatment inhibited 60% of the succinate dehydrogenase activity of chromatophores but only 8% of the activity of SDVs, indicating the membrane impermeability of phospholipase A_2. Incubation of chromatophores for 10 min with 3 mM TNBS at 5 °C and then treatment with phospholipase A_2 for 10 min and 1 h resulted in the hydrolysis of 10% and 61%, respectively, of unmodified PE. The results indicate asymmetric distributions of PE polar head groups (32–33% cytoplasmic side, 55–61% periplasmic side) and PG (73% cytoplasmic side, 14% periplasmic side) across the membrane. Also, a rapid and unidirectional transbilayer movement of PE polar head groups from the periplasmic to cytoplasmic surfaces of the membrane appears to occur during phospholipase A_2 hydrolysis on the chromatophore surfaces.

When grown anaerobically with light, the bacterium *Rhodopseudomonas sphaeroides* synthesizes a photosynthetic energy-transducing membrane system. The photosynthetic membrane is comprised of vesicular intracellular invaginations of the cytoplasmic membrane (Niederman & Gibson, 1978). Preparations of closed membrane vesicles representative of this membrane system are derived from French-pressed cell lysates (commonly termed chromatophores) (Gorchein et al., 1968; Fraker & Kaplan, 1971) or from extracts prepared by osmotic lysis of spheroplasts (Hellingwerf et al., 1975; Matsuura & Nishimura, 1977; Lommen & Takemoto, 1978). By several criteria, chromatophores are known to be oriented similarly to the in vivo intracellular vesicles and therefore inside out with respect to the cytoplasmic membrane (cytoplasmic surface out) (Scholes et al., 1969; Prince et al., 1975; Matsuura & Nishimura, 1977). In contrast, spheroplast-derived vesicles (SDVs) are oriented right side out with respect to the cytoplasmic membrane (periplasmic surface out) (Hellingwerf et al., 1975; Matsuura & Nishimura, 1977; Michels & Konings, 1978; Lommen & Takemoto, 1978; Takemoto & Bachmann, 1979). Chromatophores and SDVs can be respectively greater than 95% and 80% uniformly oriented (Lommen & Takemoto, 1978; Elferink et al., 1979; Takemoto & Bachmann, 1979). The availability of opposite and uniformly oriented vesicles with complementary exposed surfaces provides opportunities to investigate the transmembrane topography of the components comprising this membrane.

Approximately 25% of the *R. sphaeroides* photosynthetic membrane is phospholipid (Fraker & Kaplan, 1971). The phospholipids are known to assume key roles in the biosynthesis of the membrane (Lascelles & Szilagyi, 1965; Leuking et al.,

† From the Department of Biology, UMC 03, Utah State University, Logan, Utah 84322. *Received March 16, 1981.* This work was supported by National Science Foundation Grant PCM-78-08103 and the Utah Agricultural Experiment Station (Project 555).

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1978; Broglie & Niederman, 1979). However, studies of the specific roles of phospholipids in the function of the *R. sphaeroides* membrane are lacking. Similarly, nothing is known about the topographical arrangement and structure–function relationships of the phospholipids in this membrane.

Membrane-impermeable phospholipases and chemical modification reagents such as trinitrobenzenesulfonic acid (TNBS)[1] have been used to probe the transbilayer distribution of phospholipids across several membrane systems. These approaches have revealed an asymmetric distribution across the membranes of erythrocytes (Gordesky & Marinetti, 1973; Colley et al., 1973), bacteria (Rothman & Kennedy, 1977a; Paton et al., 1978; Demant et al., 1979; Kumar et al., 1979), viruses (Fong et al., 1976; Rothman et al., 1976), and eukaryotic cells (Nilson & Dallner, 1977; Sandra & Pagano, 1978). In contrast, a symmetrical distribution was shown for microsomal and golgi vesicle membranes of liver when phospholipases were used (Sundler et al., 1977). Recent evidence, however, showing a rapid, transbilayer movement of phospholipids in membranes of *Bacillus megaterium* (Rothman & Kennedy, 1977b) and sarcoplasmic reticulum (van den Besselaar et al., 1979) which could be elicited by phospholipase action complicates previous conclusions based on phospholipase hydrolyses alone.

To gain insight into the phospholipid topography of the *R. sphaeroides* photosynthetic membrane, we investigated the effects of phospholipase treatment on the phospholipids of chromatophores and SDVs and the kinetics of TNBS-labeling of these vesicles. We present data showing an asymmetric distribution of the two major phospholipids of this membrane, phosphatidylethanolamine (PE) and phosphatidylglycerol (PG). In addition, we present evidence for a rapid, transbilayer movement of PE polar head groups from the periplasmic to the cytoplasmic side of the membrane.

Materials and Methods

Chemicals. Bovine serum albumin, phosphatidylethanolamine (PE), phosphatidylglycerol (PG), phosphatidylcholine (PC), phosphatidic acid, phosphatidylserine, phosphatidylinositol, cardiolipin, 2,4,6-trinitrobenzenesulfonic acid (TNBS), lysozyme, and deoxyribonuclease I were purchased from Sigma Chemical Co. Lysophosphatidylethanolamine and lysophosphatidylglycerol were prepared from PE and PG, respectively, as described by Kates (1975). After being dried under a nitrogen stream, the lysophosphatides were dissolved in chloroform–methanol (2:1 v/v). Carrier-free [^{32}P]orthophosphate was purchased from ICN Chemical and Radioisotope Division.

Organism and Growth Conditions. Rhodopseudomonas sphaeroides (NCIB 8253) was maintained on yeast extract–malate–glutamate medium as described by Lascelles (1959). Cells were grown anaerobically with light as described by Takemoto & Bachmann (1979) except that ammonium phosphate in the growth medium was replaced by 1% (w/v) KCl, 0.42% (w/v) sodium bicarbonate, and 30 mM potassium phosphate. Cells were labeled with ^{32}P by adding 1 μCi of [^{32}P]orthophosphate/mL of growth medium at the time of cell inoculation.

Preparation of Chromatophores and SDVs. Purified chromatophores and SDVs were prepared as described by Takemoto & Bachmann (1979) except that potassium phosphate buffer (50 mM, pH 8.5) and 0.1 M KCl were used in place of Tris-HCl buffer when TNBS-modification procedures were to be performed. The vesicles were suspended to a concentration of 10 mg of protein/mL and stored at 5 °C.

Lipid Extraction and Phospholipid Analyses. One volume of vesicles (5–10 mg of protein) or cells was extracted with 20 volumes of chloroform–methanol (2:1 v/v) as described by Lascelles & Szyilagi (1965). The extract was dried in a rotary evaporator at 60 °C and dissolved in a small volume (30–50 μL) of chloroform–methanol (2:1 v/v) for application to thin-layer chromatography plates. Lipid extracts (30 μL) were applied to silica gel (60 G, Merck) thin-layer chromatography plates (20 × 20 cm) prepared according to the methods of Skipski & Barclay (1969). Both one- and two-dimensional chromatography were performed with solvents chloroform–methanol–water (65:24:4 v/v) or chloroform–methanol–4 M ammonia (65:35:5 v/v) in the first dimension and chloroform–methanol–acetic acid (65:24:4 v/v) in the second dimension. Phospholipids were detected by using potassium dichromate in sulfuric acid (Skipski & Barclay, 1969) or molybdenum blue reagent (Dittmer & Lester, 1964). For quantitation of phospholipids, [^{32}P]phospholipid spots were scraped off the plates and counted by liquid scintillation methods. Individual phospholipids were identified by cochromatography with commercial phospholipid standards.

TNBS Modification. ^{32}P-Labeled chromatophores or SDVs (5 mg) protein were incubated with TNBS (0.1 mM to 5 mM as designated), 0.1 M KCl, and 50 mM potassium phosphate buffer, pH 8.5, in a total volume of 1 mL between 5 and 37 °C. The mixture was occasionally and gently stirred. At designated times, the reaction was stopped with the addition of 0.1 mL of ice-cold trichloroacetic acid (30% w/v) and 0.125 mg of bovine serum albumin.

For estimates of TNBS permeability, ^{32}P-labeled chromatophores (5 mg of protein) were treated with sodium dodecyl sulfate (1% w/v) or Triton X-100 (1% w/v) in 5% (w/v) sodium bicarbonate for 10 min at 25 °C. No detergents were added to control chromatophore samples. The mixtures were cooled to 5 °C, and TNBS was added to a final concentration of 3 mM. The mixtures were incubated further for 10 min at 5 °C. The reactions were terminated as described above.

Phospholipase A_2 Hydrolysis. ^{32}P-Labeled chromatophores and SDVs (5 mg of protein) were treated with 1–25 units of phospholipase A_2 (*Naja naja*) (EC 3.1.1.4) (Sigma), 1 mM $CaCl_2$, and 10 mM Tris-HCl, pH 8.5 in a total volume of 1 mL. The mixture was initially shaken vigorouly on a vortex mixer for 30 s and then incubated at 37 °C on a rotary shaker (300 rpm) for a designated time. The reaction was stopped with the addition of 0.1 mL of 1 M EDTA, pH 8.5. A unit of phospholipase A_2 hydrolyzed approximately 1 μmol of phosphatidylcholine per min.

Effect of Phospholipase A_2 Treatment on Succinate Dehydrogenase (EC 1.3.99.1). Chromatophores and SDVs (5 mg of protein) were treated with 5 units of phospholipase A_2 as described above for 5, 15, 45, 60, and 75 min. The samples were then assayed directly for succinate dehydrogenase activities with phenazine methosulfate and 2,6-dichlorophenolindolphenol as electron acceptors, as described by Takemoto & Bachmann (1979).

Measurement of Transbilayer Movement of PE. ^{32}P-Labeled chromatophores (5 mg of protein) were incubated with 3 mM TNBS, 0.1 M KCl, and 50 mM potassium phosphate buffer, pH 8.5, in a total volume of 1 mL for 10 min at 5 °C. The mixture was then diluted in 10 volumes of the above potassium phosphate buffer and then centrifuged at 150000*g*

[1] Abbreviations used: SDVs, spheroplast-derived vesicles; PE, phosphatidylethanolamine; PG, phosphatidylglycerol; TNBS, trinitrobenzenesulfonate; PC, phosphatidylcholine; Tris, tris(hydroxymethyl)-aminomethane; EDTA, ethylenediaminetetraacetic acid.

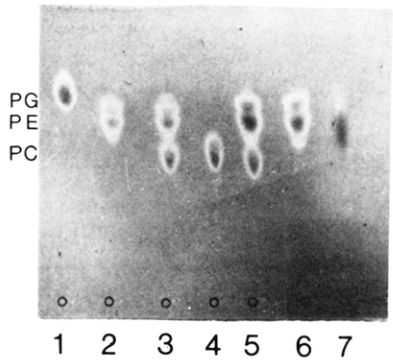

FIGURE 1: Thin-layer silica gel chromatogram (single dimension) of authentic samples of PG (1), PC (4), and PE (7), SDV (2) and chromatophore (6) lipid extracts, SDV extract plus PC (3) and chromatophore extract plus PC (5). Vesicle extracts (30 μL) from 5 mg of vesicle protein with or without 120 μg of PC or 120 μg of authentic phospholipids (in 30 μL chloroform–methanol (2:1 v/v)) were applied to the plate. The plate was stained with molybdenum blue reagent (Dittmer & Lester, 1964).

Table I: Phospholipid Composition of Whole Cells, Chromatophores, and SDVs[a]

	percent of total phospholipids[b]			
sample	PE	PG	PC	unidentified phospholipids
whole cells	65.5 ± 0.8	30.0 ± 0.7	2.2 ± 0.3	2.3 ± 0.8
chromatophores	62.0 ± 1.0	34.0 ± 1.0	nd[c]	3.7 ± 0.6
SDVs	62.0 ± 0.9	33.0 ± 0.9	nd	4.7 ± 0.4

[a] Values based on six determinations. [b] Total radioactivity (100%) was calculated by adding values of all detected phospholipids and ranged between 5500 and 8000 cpm/30 μL of applied sample. [c] Not detected.

for 1 h in a Beckman 50.2 Ti rotor and L5-65 centrifuge at 5 °C. The pellet was carefully suspended in buffer (10 volumes) and the centrifugation repeated. The modified chromatophores were suspended in 1 mL of 10 mM Tris-HCl, pH 8.5, and 1 mM $CaCl_2$ containing 5 units of phospholipase A_2. After 10-min or 1-h incubation at 37 °C, the reaction was terminated as described above, and the lipids were extracted for separation and analyses.

Protein Assay. Protein levels of vesicles were determined by using the method of Markwell et al. (1978), with bovine serum albumin as standard.

Results

Phospholipid Identification and Composition. Phospholipids of purified chromatophores and SDVs were identified and quantitated on thin-layer silica gel plates after single or two-dimensional chromatography. The single-dimensional system permitted adequate resolution of PE, PG, and PC and of these lipids occurring in the samples (Figure 1). The chromatographic patterns resembled those of Gorchein (1968). Molybdenum blue staining of the plates revealed that PE and PG were the predominant phospholipids of the vesicles. Quantitative phospholipid composition analyses of ^{32}P-labeled cells, chromatophores, and SDVs are shown in Table I. In all samples, PE and PG comprised 95% or more of the total phospholipids. Although 2% of the total cellular phospholipid was PC, PC was not detected in the purified vesicle preparations. Authentic PC was resolved on the chromatograms even when it was initially added to chromatophore or SDV lipid extracts before application to the plates (Figure 1). Thus, the inability to detect PC in the extracts does not appear to result from substances in the extracts (e.g., polybeta-

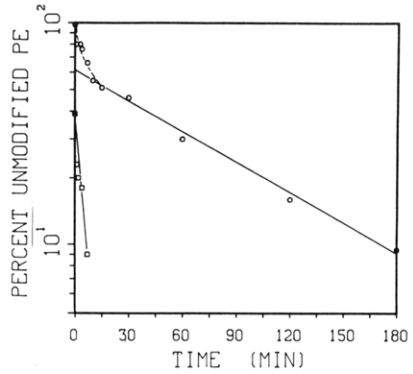

FIGURE 2: Kinetics of TNBS modification of chromatophore PE at 5 °C. The data of Figure 1 were plotted on a semilogarithmic graph to reveal an initial phase (O- - -O) and a linear slow phase (O—O) of modification. The slow phase was extrapolated to zero time and subtracted from the initial phase to show the kinetics of a fast-reacting PE pool (□—□). The slopes and intercepts of the slow and fast-reacting components were determined by least-squares fit analyses.

hydroxybutyrate) which might interfere with the chromatographic separation of lipids (Gorchein, 1968). Phosphatidylserine, phosphatidylinositol, phosphatidic acid, and cardiolipin were also not detected. Between 2% and 5% of the phospholipids were not identified.

TNBS Modification of PE. TNBS couples with primary amino groups at pH 8 and is relatively impermeable to natural and artificial membranes (Litman, 1973; Roseman et al., 1975; Rothman & Kennedy, 1977a).

Treatment of ^{32}P-labeled chromatophores with TNBS (0.1–5 mM) for various times (0–3 h) at several temperatures (5–37 °C) yielded a single radioactive yellow spot on thin-layer chromatograms corresponding to the trinitrophenylated derivative of PE. The modified PE was clearly resolved from unmodified PE (Rothman & Kennedy, 1977a). With 3 mM TNBS, the kinetics of modification differed at 5 and 25 °C. Fifty percent modification of PE occurred in 2 min at 25 °C and in 22 min at 5 °C. About 95% of the PE reacted with TNBS after 180-min incubation at both temperatures. The reaction kinetics at 5 °C were biphasic, as revealed by plotting the data as percent of unreacted PE on a logarithmic scale vs. incubation time (Figure 2). An initial phase representing modification of both fast- and slow-reacting pools of PE was followed by a phase representing the slow-reacting pool alone. The reaction kinetics of the fast-reacting pool were calculated by subtracting the slow phase (extrapolated to zero time) from the initial phase. The calculated fast phase was a straight line, indicating the biphasic nature of the overall reaction kinetics. Similar kinetics have been observed with *B. megaterium* cytoplasmic membrane (Rothman & Kennedy, 1977a). The fast-reacting pool comprised 33% of the total chromatophore PE and had a reaction half-time of 4 min. The slow-reacting pool comprised 61% of the total and had a reaction half-time of 59 min.

These results indicate that 33% of the PE amino groups are readily accessible to TNBS at 5 °C before significant penetration into the membrane, and this fraction is most reasonably located close to or on the outer surface of the chromatophores. The remaining PE amino groups are located close to the inside surface or sequestered within the membrane and become modified only after TNBS penetrates the membrane.

Similar experiments were performed with TNBS and SDVs. Again, biphasic kinetics of PE modification were observed (Figure 3). A fast-reacting pool of PE comprised 55% of the total PE and had a half-reaction time of 7 min. A slow-reacting pool comprised 32% of the total and had a half-reaction

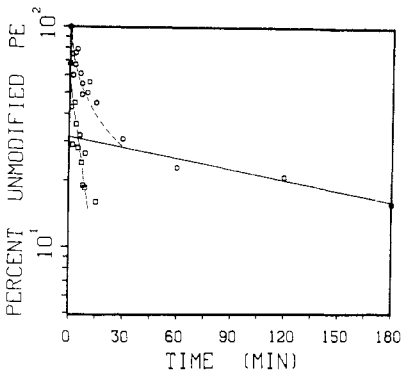

FIGURE 3: Kinetics of TNBS modification of SDV PE at 5 °C. The data were obtained and treated as described in Figure 2. Total radioactivity (100%) (PE plus modified PE) was approximately 4000 cpm/30 μL of applied extract.

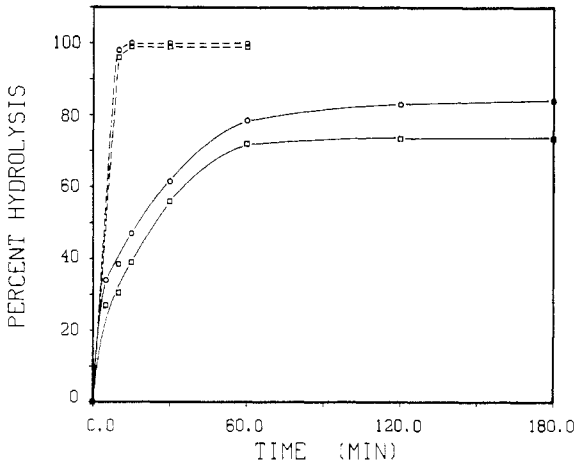

FIGURE 4: Time course of the effect of phospholipase A_2 on chromatophore PE and PG. Chromatophores (5 mg of protein) were treated with 5 units (—) or 25 units (----) of enzyme. PE (○) and PG (□) hydrolyses were analyzed by thin-layer chromatography. Data are given as percent of total PE or PG converted to the corresponding lysophosphatide. Total (100%) PE and PG were approximately 3500 and 1800 cpm/30 μL of extract, respectively.

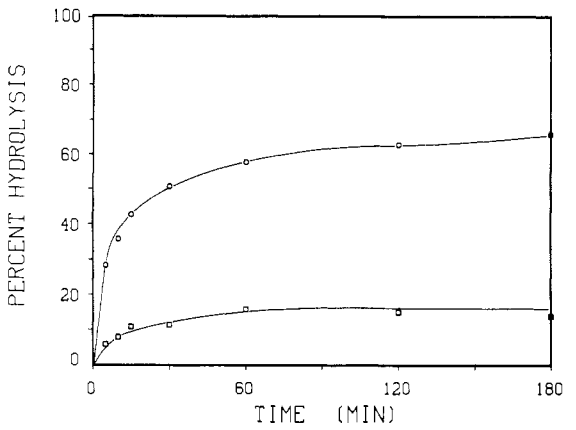

FIGURE 5: Time course of the effect of phospholipase A_2 on PE (○) and Pg (□) of SDVs. SDVs (5 mg of protein) were treated with 5 units of enzyme. The data are presented as described in Figure 4. Total (100%) PE and PG were approximately 4400 and 2500 cpm/30 μL of extract, respectively.

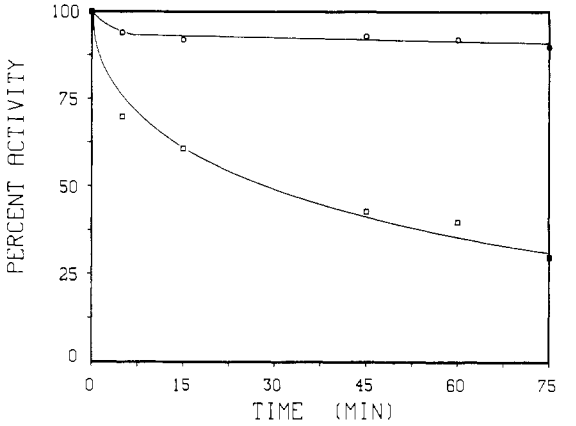

FIGURE 6: Effect of phospholipase A_2 treatment on succinate dehydrogenase activities of chromatophores (□) and SDVs (○). The data are presented as percent of activity measured with untreated vesicles. Specific activities of untreated chromatophores and SDVs (100%) were 0.81 and 0.95 μmol of 2,6-dichlorophenolindophenol reduced per min per mg of protein.

time of 173 min. Thus, 55% of the PE amino groups appears immediately accessible on the outer surfaces of the SDVs. This distribution roughly complements the PE distribution observed with the oppositely oriented chromatophores.

Permeability of TNBS. The above kinetic analyses indicate that the *R. sphaeroides* photosynthetic membrane is relatively impermeable to TNBS at a concentration of 3 mM at 5 °C and short incubation times. Further support for TNBS impermeability was provided by observations of TNBS reaction with chromatophores pretreated with detergents to disrupt the membrane. Chromatophores treated with 1% (w/v) sodium dodecyl sulfate or 1% (w/v) Triton X-100 at 25 °C and then incubated with 3 mM TNBS at 5 °C for 10 min showed 65% increases in the fraction of total PE modified when compared to controls not treated with detergents.

Phospholipase A_2 Treatment of Chromatophores and SDVs. Radioactive ^{32}P-labeled chromatophores were treated with phospholipase A_2 (1 unit/mg of protein) for various time intervals at 37 °C (Figure 4). The corresponding lysophosphatides of PG and PE were clearly resolved by single-dimensional chromatography. Approximately 73% and 77% of the total PG and PE, respectively, were converted to their lysophosphatides after 1-h incubation. No further hydrolysis of PG was observed with further incubation times up to 3 h. Hydrolysis of PE, however, continued after the first hour until 84% of this phospholipid was hydrolyzed after 3 h. With a 5-fold higher level of enzyme, complete hydrolysis of both phospholipids was achieved after 10-min incubation, indicating possible membrane disruption with this high level of enzyme. In contrast, a 5-fold lower level of enzyme gave less than 5% hydrolysis of PE and PG after 1-h incubation.

Similar phospholipase A_2 treatments were performed with ^{32}P-labeled SDVs (Figure 5). Nearly 60% of the PE was hydrolyzed after 1-h incubation, and this increased further to 66% after 3-h incubation. Approximately 14% of the PG was hydrolyzed after 1 h, and no further increase occurred up to 3-h incubation.

Assuming impermeability of phospholipase A_2 in the membrane, the above results indicate that 77% and 60% of the total PE polar head groups are accessible to the enzyme on the cytoplasmic and periplasmic membrane surfaces, respectively, during a 1-h incubation period. The apparent noncomplementary distribution of PE polar head groups across the membrane may be explained by a rapid, transbilayer movement of PE polar head groups to the chromatophore outer surface during phospholipase treatment (see below). In contrast, the results reveal a roughly complementary distribution of PG polar head groups across the membrane, with 73% and 14% on the cytoplasmic and periplasmic surfaces, respectively.

Permeability of Phospholipase A_2. The difference in the extent of PG hydrolysis with chromatophores and SDVs with 1 unit of phospholipase A_2 per mg of vesicle protein suggests

Table II: Transbilayer Movement of PE in Chromatophores

time of phospholipase treatment	phospholipid	radioactivity (cpm)[a]	percent of total
10 min	lyso-PE[b]	423	10
	PE	2324	55
	Tnp-PE[c]	1056	25
	lyso-Tnp-PE[d]	422	10
1 h	lyso-PE	1910	61
	PE	424	14
	Tnp-PE	559	18
	lyso-Tnp-PE	222	7

[a] Counts per minute. [b] Lysophosphatide of PE. [c] Trinitrophenylated derivative of PE. [d] Lysophosphatide of Tnp-PE.

that the enzyme does not penetrate the membrane at this level. The impermeability of phospholipase A_2 was further suggested by measurements of the effects of enzyme treatments on the succinate dehydrogenase activities of chromatophores and SDVs (Figure 6). Succinate dehydrogenase was shown previously to be located on the outside surface of chromatophores (or inside surface of SDVs) and can be measured from either side of the membrane by using phenazine methosulfate and 2,6-dichlorophenolindophenol as electron acceptors (Takemoto & Bachmann, 1979). After 1-h incubation of chromatophores with phospholipase A_2 (1 unit/mg of protein), approximately 60% of succinate dehydrogenase activity was inhibited. Less than 8% of the activity of SDVs was inhibited after 1 h of enzyme treatment. These results indicate that (1) succinate dehydrogenase activity is dependent upon the native configuration of phospholipids closest to the outer surfaces of the chromatophores and (2) phospholipase A_2 does not penetrate the membrane under the incubation conditions used.

A further indication that phospholipase A_2 did not penetrate the membrane at this level was the sedimentation properties of treated chromatophores. Chromatophores treated with enzyme (1 unit/mg of protein) for 1 h at 37 °C were quantitatively pelleted after centrifugation at 150000*g* for 1 h. Thus, the structural integrity of the chromatophores was apparently maintained, suggesting that degradation as a result of penetration did not occur.

Transbilayer Movement of PE in Chromatophores. The TNBS modification results indicate that 33% of the total membrane PE amino groups are situated near the outer surface of chromatophores. However, the phospholipase hydrolysis results show that nearly 80% of the PE head groups are on the chromatophore surface with 1-h incubation at 37 °C. The discrepancy may be due to a transbilayer displacement of the PE head groups from the inner chromatophore surfaces to the outer surfaces during enzyme hydrolysis. Analogous transbilayer movements of phospholipids have been observed in other natural membranes (Rothman & Kennedy, 1977b; van den Besselaar et al., 1979). To test this possibility, we incubated ^{32}P-labeled chromatophores with TNBS (3 mM) for 10 min at 5 °C to modify predominantly the outer surface PE polar head groups (Figure 2). The chromatophores were then washed and incubated with phospholipase A_2 for 10 min or 1 h at 37 °C. The lipids were extracted and analyzed for the various forms of PE on thin-layer chromatograms (Table II). Of the total PE 10% and 61% were converted to the lysophosphatide of PE with 10-min and 1-h enzyme treatments, respectively, indicating that polar head groups of these fractions were originally located on the inner surfaces of the chromatophores (inaccessible to TNBS) and subsequently redistributed to the outer surfaces during hydrolysis. Most of the TNBS-modified PE (70%) was not hydrolyzed, indicating that it may be a less efficient substrate for phospholipase A_2 than unmodified PE.

Table III: Distribution of PE and PG across the *R. sphaeroides* Photosynthetic Membrane

phospholipid	percent distribution: cytoplasmic side	percent distribution: periplasmic side	method of determination
PE	32–33	55–61	TNBS modification
PG	73	14	phospholipase A_2 hydrolysis

Discussion

Estimates of the distribution of PE and PG across the *R. sphaeroides* photosynthetic membrane are summarized in Table III. We conclude that both phospholipids are asymmetrically distributed across the membrane with PE predominantly located on the periplasmic side and PG predominantly on the cytoplasmic side. The estimated figures are probably conservative indications of the degree of asymmetry since the vesicles used are not absolutely uniform in orientation (Lommen & Takemoto, 1978; Elferink et al., 1979; Takemoto & Bachmann, 1979). Assuming that PE comprises 62% and PG 34% of the total phospholipid in this membrane (Table I), the above estimated distributions will result in the occurrence of 41% and 45% of the total phospholipid in the periplasmic and cytoplasmic half-bilayer leaflets, respectively.

Between 6% and 13% of the total membrane PE and 13% of the PG were not accessible to TNBS modification and phospholipase A_2 hydrolysis, respectively. These fractions may reflect the occurrence of phospholipids with polar head groups sequestered in the interior of the membrane and possibly associated with hydrophilic portions of integral proteins. The small fraction of unhydrolyzed PG may also represent PG molecules which are inaccessible because of packing restraints in local, crystalline regions of the membrane (Bevers et al., 1977). However, this is less likely since the phospholipase hydrolyses were performed at 37 °C.

The functional significance of the asymmetric distribution of PG and PE is unknown. However, the predominance of PG on the cytoplasmic surface may be important in considering electron-transport function and other processes occurring in association with this surface. At least 70% of the phospholipid polar head groups on or near the cytoplasmic surface are those of PG which would provide a large concentration of negative charges on this surface. Overfield & Wraight (1980) have recently demonstrated the importance of surface charge imparted by phospholipid in influencing electron-transfer interactions of peripheral proteins with integral protein complexes. Birrell et al. (1978) have demonstrated a preferential binding of negatively charged lipid polar head groups to chromatophore proteins (predominantly light-harvesting protein) in *R. sphaeroides*. This latter study suggests a specific role for PG, the major negatively charged phospholipid, in lipid–protein interactions of this membrane. Further studies on the nature of specific phospholipid–protein associations and the roles of individual phospholipids are required to assess the full significance of the observed asymmetry.

The occurrence of aminophospholipid polar head groups (e.g., PE) predominantly at the periplasmic surface differs from the distribution seen with other bacterial membranes. Studies on the cytoplasmic membrane of various *Bacillus* species (Rothman & Kennedy, 1977; Bishop et al., 1977; Paton

et al., 1978) and *Mycobacterium phlei* (Kumar et al., 1979) indicate a preferential location of PE on the cytoplasmic side in these species. A similar preferential location of aminophospholipids on cytoplasmic surfaces seems to occur generally for animal cell membranes (Opde Kamp, 1979). Interestingly, Shimada & Murata (1976) have reported a preferential location of PE on the inner surfaces of chromatophores (periplasm inside) isolated from the photosynthetic bacterium, *Chromatium vinosum*. Whether or not this particular distribution is unique to the photosynthetic bacteria as a group must still be determined with investigations of other species.

Preliminary evidence for a transbilayer movement of PE polar head groups from the periplasmic to the cytoplasmic surfaces with phospholipase treatment is presented. The movement appears relatively rapid, with a half-time of about 35 min (Table II) when compared to PE flip-flop rates measured in pure lipid vesicles (Roseman et al., 1975). PE movement in the opposite direction from the cytoplasmic to periplasmic surfaces apparently does not occur since the extent of hydrolysis by phospholipase (1-h treatment) (Figure 5) and the extent of TNBS modification (Figure 2) on the SDV surfaces were approximately the same (60% and 55%, respectively). Similarly, the roughly complementary distribution of PG polar head groups on the cytoplasmic (Figure 4) and periplasmic (Figure 5) surfaces (73% and 14%, respectively) suggests no appreciable transbilayer movement of PG molecules. Thus, the rapid, transbilayer movement appears specific for PE and unidirectional.

Whether or not a rapid, transbilayer movement of PE (or PG) occurs in the unperturbed membrane and is related to the phospholipase-induced movement needs to be determined. The phospholipid content of the *R. sphaeroides* photosynthetic membrane is known to fluctuate dramatically during growth (Lueking et al., 1978) and to influence the incorporation and function of electron-transport components in the membrane (Wraight et al., 1978). Conceivably, a rapid transbilayer movement of PE which comprises greater than 60% of the total phospholipids could play an important role in the synthesis and function of this membrane.

Our inability to detect PC in purified preparations of chromatophores and SDVs is not consistent with previous reports of this phospholipid occurring in membrane preparations of *R. sphaeroides* (Gorchein, 1968; Russell & Harwood, 1979). However, Haverkate et al. (1965) reported very low levels of PC in this organism. Gorchein (1968) noted the wide variability in reported PC levels in *R. sphaeroides* as well as levels of other phospholipids. The author suggested several possibilities for the discrepancies, including differences in growth conditions, cell strains employed, and substances in the lipid extracts which might interfere with chromatographic separation of lipids. This last possibility does not appear to apply to the presently reported studies (Figure 1). We have estimated that PC comprises 2% of the total cellular phospholipid, suggesting that it is perhaps localized in some other cell fraction such as the outer cell enevelope.

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Effect of Carboxyl Group Modification on Redox Properties and Electron Donation Capability of Spinach Plastocyanin†

Kent O. Burkey and Elizabeth L. Gross*

ABSTRACT: Spinach plastocyanin was chemically modified by using a water-soluble carbodiimide to form an amide bond between a protein carboxyl group and one amino group of ethylenediamine. On the average, four plastocyanin carboxyl groups were replaced with positively charged amino groups. Modified plastocyanin facilitated high rates of electron donation to the oxidized P700 reaction center of photosystem I particles in the absence of cations. Control plastocyanin was totally inactive in the absence of divalent cations due to charge repulsion between the negatively charged plastocyanin and photosystem I proteins. The K_m for the binding of modified plastocyanin to photosystem I particles was 2.1 μM compared to 36.5 μM for control plastocyanin in the presence of $MgCl_2$. Therefore, chemical modification was more effective than charge shielding by cations in facilitating the binding of plastocyanin to photosystem I. Chemical modification also increased the midpoint redox potential of plastocyanin from +380 to +420 mV, which indicated an alteration of the copper environment. Ethylenediamine was covalently attached to tryptic peptides from plastocyanin that contained amino acid residues 42–45, 59–61, and 68 which are highly conserved glutamic acid and aspartic acid residues. Chemically altering the charge on these residues changed the activity of plastocyanin which indicates that these amino acids are involved in the ionic regulation of the plastocyanin–PSI interaction. Because chemical modification also altered the environment of the chromophore, certain of these amino acids must be located near the copper site.

Plastocyanin is a small copper protein which acts as an electron carrier in photosynthetic electron transport (Boulter et al., 1977). Reconstitution experiments (Plesnicar & Bendall, 1973; Gorman & Levine, 1966) as well as kinetic evidence (Haehnel et al., 1980) indicate that plastocyanin accepts electrons from cytochrome *f* and donates electrons to $P700^+$.[1]

Recent studies (Tamura et al., 1980; Haehnel et al., 1980) with broken spinach chloroplasts have shown that salts stimulate the rate of plastocyanin electron donation to $P700^+$. Their conclusion was that salts increase the local concentration or accessibility of plastocyanin to the site of P700 on the membrane by causing a shift in the negative surface potential toward neutrality. Both spinach plastocyanin (Boulter et al., 1977) and the thylakoid membrane (Nakatani & Barber, 1980) are negatively charged at neutral pH. By screening the negative charges on these entities, salts reduce electrostatic repulsion between them which allows plastocyanin to donate electrons to $P700^+$ more readily.

This type of electrostatic interaction also exists between spinach plastocyanin and isolated PSI[1] which is also a negatively charged protein complex (Satoh & Butler, 1978; Sieferman-Harms & ninnemann, 1979). Lien & San Pietro (1979) observed a cation stimulation of $P700^+$ reduction by plastocyanin when isolated PSI particles were examined. This stimulation was later found to be the result of lowering the K_m for plastocyanin binding (Davis et al., 1980). Therefore, salts increase the accessibility of plastocyanin to P700 when the isolated components are recombined.

Recently, Burkey & Gross (1981) studied the electrostatic interaction between spinach plastocyanin and isolated PSI particles by altering the net charge of PSI through chemical modification. The modification reaction involved reacting the PSI complex with a water-soluble carbodiimide in the presence of ethylenediamine (see eq 1)

$$\text{protein–COO}^- + :\text{NH}_2(\text{CH}_2)_2\text{NH}_3^+ \xrightarrow[\text{pH 6.0}]{\text{carbodiimide}} \text{protein–CONH}(\text{CH}_2)_2\text{NH}_3^+ \quad (1)$$

(Means & Feeney, 1971). The reaction produced a large number of modified carboxyl groups on PSI which resulted in a positively charged PSI complex with the P700 reaction center intact. The modified PSI was capable of high rates of $P700^+$ reduction by plastocyanin in the absence of salts. Salts were no longer required because the negatively charged plastocyanin was electrostatically attracted to the positively charged modified PSI. This demonstrated that protein charge

† From the Department of Biochemistry, The Ohio State University, Columbus, Ohio 43210. *Received March 25, 1981.* This work was supported by Grant No. PCM 7806969 AQ1 from the National Science Foundation.

[1] Abbreviations used: chl, chlorophyll; PSI, photosystem I particles; P700, reaction center chlorophyll of photosystem I which absorbs maximally at 700 nm; $P700^+$, oxidized P700; $NaDodSO_4$, sodium dodecyl sulfate; Temed, *N,N,N′,N′*-tetramethylethylenediamine; DEAE, diethylaminoethyl; Tris, tris(hydroxymethyl)aminomethane; TPCK, tosylphenylalanine chloromethyl ketone; CM, carboxymethyl.

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