Vol. 95, No. 3, 1980 BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS
August 14, 1980 Pages 1049-1055

EFFECT OF VARIOUS WATER-SOLUBLE COMPONENTS OF PHOSPHOLIPIDS ON THE PHOTOOXIDATION OF CHLOROPHYLLIN

William Stillwell and Susan Karimi

Purdue University, School of Science
Biology Department
1201 East 38th Street, Indianapolis, Indiana 46205

Received June 23,1980

Summary: Photooxidation of water-soluble chlorophyllin was measured in the presence of glycerol, phosphate, choline, phosphorylcholine, ethanolamine, phosphorylethanolamine and liposomes made from phosphatidylcholine and phosphatidylethanolamine. The choline containing compounds protected chlorophyllin from destruction while the ethanolamines enhanced degradation. The difference in activity between the two families of compounds is related to the availability of their protons. These experiments provide a further indication that in the thylakoid phosphatidylcholine may protect chlorophyll from photooxidation.

In stark contrast to its apparent stability in vivo, chlorophyll is known to rapidly degrade by photooxidation in organic solvents (1-3). The components of the thylakoid involved in protecting chlorophyll from degradation as well as their mechanisms of action have yet to be elucidated (4). In the 1950's Stanier (5-7) and others (8) demonstrated that both in vivo and in vitro chlorophyll can be protected from photooxidation by carotenoids. Indeed antioxidation has been ingrained in the literature as a major role for carotenoids in photosynthesis (9). More recently, Stillwell and Tien (10) reported a substantial protection from photobleaching of chlorophyll by phospholipids in chloroform. They showed that protection was provided by the phospholipid polar head group as a series of phosphatidylcholines protected chlorophyll much more efficiently than did a similar series of phosphatidylethanolamines or asolectin. Protection was independent of the chain length and degree of unsaturation of the esterified fatty acids. Also, no protection was afforded by any mono- or diacylglycerols tested.

The observation that phosphatidylcholine could protect chlorophyll from photooxidation was later substantiated by Van Hasselt et al. (4) who investigated the effect of several compounds including monogalactosyldiglyceride, phosphatidylcholine and β-carotene on the rate of degradation of chlorophyll a in acetone. They too concluded that the polar head group of phosphatidylcholine can severely decrease the susceptibility of chlorophyll to photooxidation.

0006-291X/80/151049-07$01.00/0

In the study presented here we are taking the problem a step further by asking the question: "Which of the components of the phospholipid head group are responsible for protecting chlorophyll from oxygen and light?" In a search for answers to this question we decided to use water-soluble chlorophyllin (the saponification product of chlorophyll). This molecule is very water-soluble, has an intact porphyrin ring but is missing the phytol chain, and absorbs light strongly at 630 nm. Chlorophyllin has been shown to bind to the polar head group region of artificial lipid bilayers (11) and so would probably be found in a similar position on the membrane surface as chlorophyll (12-14). By replacing the almost totally water-insoluble chlorophyll with chlorophyllin, we found we could readily measure in aqueous solution the effect of glycerol, phosphate, choline, phosphorylcholine, ethanolamine and phosphorylethanolamine on chlorophyllin photooxidation. From these studies we have arrived at some conclusions concerning the nature of protection from photooxidation of chlorophyll by phosphatidylcholine.

MATERIALS AND METHODS: Chlorophyllin was purchased from Pflatz and Bauer. Choline, phosphorylcholine, ethanolamine phosphorylethanolamine, phosphatidylcholine and phosphatidylethanolamine were provided by Sigma Chemical Company. All other chemicals were reagent grade and were used without further purification.

Since chlorophyllin was found to degrade even when stored in the dark at 3° C, all solutions were made fresh immediately before being tested. Chlorophyllin concentration was always set to give an initial absorbance at 630 nm of 1.0. This corresponded to a 1.54 mM solution of chlorophyllin. Samples, placed in cuvettes, were irradiated from the top with actinic light from a light pipe (Fiber-Lite High Intensity Illuminator, Model 170-D, on the top setting). The average light intensity in the sample cuvette was 640 watts M^{-2}. Irradiations were conducted at 25° C in a dark, sealed chamber to eliminate the effect of extraneous light. The cuvette was removed ater 0, 1, 3, 5, 10, 15, 20 and 25 minutes of irradiation and the absorbance at 630 nm measured on a Beckman DBGT Spectrophotometer set on the expanded scale. Liposomes were made by the sonication method of Bangham (15) from phosphatidylcholine (0.2 g/20 ml of water) and phosphatidylethanolamine (0.189 g/20 ml of water). Sonication time was 15 minutes on the top power setting of a Heat Systems-Ultrasonic Model W-220 F Cell Disruptor. Four ml of this concentrated liposome solution was then diluted to 20 ml and this slightly milky suspension was added to both the sample and reference cuvettes before chlorophyllin addition to the sample cuvette. Again the initial chlorophyllin absorbance was set at 1.0.

RESULTS: The rate of degradation for chlorophyllin in aqueous solvent at pH 7.0 was accurately determined spectrophotometrically. The initial degradation rate, determined for the first 5 minutes, was $1.20 \pm .03 \times 10^{-4}$ mg/ml/min. This number, the average of many experiments, was taken as the control and variations from this number measured in the presence of various ingredients and reported in Tables 1, 2 and 3 and Figure 1.

TABLE 1. Photooxidation of chlorophyllin at pH 7.0 in the presence of various water-soluble components. Degradation rates for chlorophyllin were determined for the initial 5 minutes and the data reported as mg of chlorophyllin degraded/ml/minute X 10^{-4}. The concentration of all water soluble components is 10 mM except chlorophyllin which is 1.54 mM.

Compound	Degradation Rate of Chlorophyllin mg/ml/min X 10^{-4}
control (no additions)	1.20
glycerol	1.26
phosphate	1.26
phosphorylcholine	1.08
phosphorylethanolamine	1.51
choline	1.12
ethanolamine	2.27

Table 1 reports the effect of 10 mM quantities of various components of phospholipid head group on the initial degradation rate of chlorophyllin at pH 7.0. Glycerol and phosphate had no substantial effect although a slight increase in degradation was measured. Some protection was noted for choline and a larger protection for phosphorylcholine. Rapid degradation occurred with ethanolamine and phosphorylethanolamine. These results,

TABLE 2. Photooxidation of chlorophyllin at pH 7.0 in the presence of different amounts of choline, phosphate and phosphorylcholine. Degradation rates are expressed as mg of chlorophyllin degraded/ml/minute X 10^{-4}.

Compound	Concentration (mM)	Degradation Rate of Chlorophyllin mg/ml/min X 10^{-4}
choline	0.0	1.20
	10.0	1.12
	20.0	1.01
	100.0	0.34
phosphate	0.0	1.20
	1.0	1.20
	10.0	1.26
	20.0	1.26
	100.0	1.68
phosphorylcholine	0.0	1.20
	10.0	1.08
	20.0	0.99
	100.0	(insoluble)

TABLE 3. Photooxidation of chlorophyllin in the presence of 10 mM phosphate or 10 mM phosphorylcholine as a function of pH. Degradation rates are expressed as mg of chlorophyllin degraded/ml/minute X 10^{-4}.

Compound	pH	Degradation Rate of Chlorophyllin, mg/ml/min X 10^{-4}
control (no addition)	7.0	1.20
phosphate	3.0	2.35
	7.0	1.26
	11.0	0.15
phosphorylcholine	7.0	1.18
	8.0	0.67
	9.0	0.59
	10.0	0.42

obtained in aqueous solution, are in agreement with those previously reported on the degradation of chlorophyll in chloroform in the presence of phosphatidylcholines and phosphatidylethanolamines (10). Phosphatidylcholine provides more protection from photooxidation than does phosphatidylethanolamine.

The effect of increasing concentrations of components of the phosphatidylcholine head group on chlorophyll degradation at pH 7.0 is reported in Table 2. Degradation rates decreased with increasing quantities of choline and phosphorylcholine but increased with

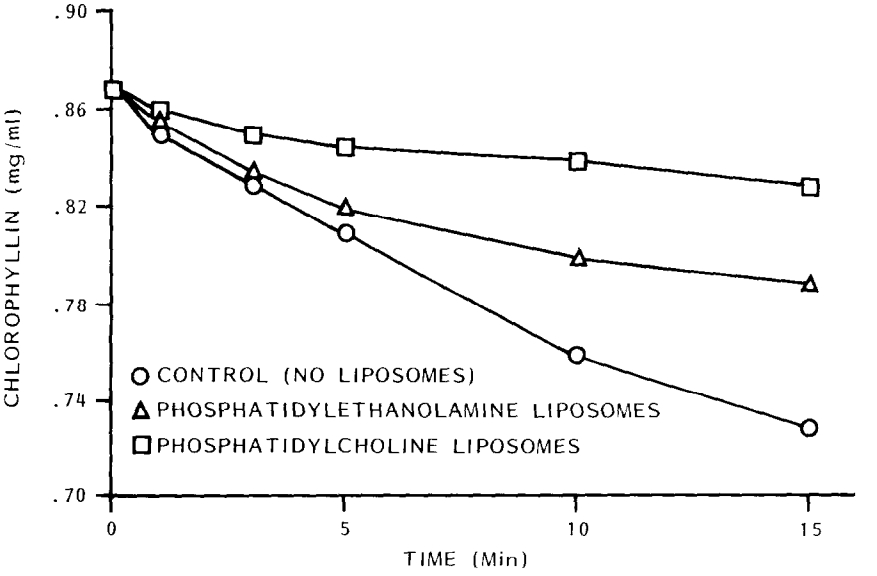

FIGURE 1. Photooxidation of chlorophyllin in the presence of phosphatidylcholine liposomes, phosphatidylethanolamine liposomes and no liposomes (control).

increasing amounts of phosphate. Very large amounts of choline (100 mM) provided substantial protection for chlorophyllin from photooxidation. (Phosphorylcholine was not soluble at 100 mM at pH 7.0).

Chlorophyll degradation is known to be enhanced by acids (1). Therefore the rate of chlorophyllin photooxidation was followed as a function of pH. The data is presented in Table 3. Degradation of chlorophyllin was substantial with 10 mM phosphate at pH 3.0 but decreased rapidly as the pH was increased to 7.0 and was almost totally suppressed at pH 11.0. A similar increase in protection of chlorophyllin photooxidation in the presence of 10 mM phosphorylcholine was measured from pH 7.0 to 10.0. (Phosphorylcholine is not soluble at 10 mM below pH 7.0).

The last set of experiments shown in Figure 1 measured the rate of chlorophyllin degradation in the presence of liposomes made from phosphatidylcholine or phosphatidylethanolamine. Phosphatidylcholine liposomes greatly enhanced the protection from photooxidation while the effect of phosphatidylethanolamine was substantially less. These results are consistent with the previously reported photooxidation protection of chlorophyll in organic solvents (4, 10).

DISCUSSION: The results presented above confirm the previous reports of Stillwell and Tien (10) and Van Hasselt et al. (4). Using water-soluble chlorophyllin and liposomes, phosphatidylcholine is shown to greatly protect the pigment from photooxidation. With this system, phosphatidylcholine is shown to protect chlorophyllin to a much greater extent than is phosphatidylethanolamine. The noticeable difference in protecting ability between phosphatidylcholine and phosphatidylethanolamine implies that the polar head group is the active component of the phospholipid. The effect of phosphatidylcholine on preventing photooxidation also confirms that there is a strong interaction between the exposed polar head group on the liposome surface and the porphyrin ring of chlorophyllin. The conclusion that chlorophyllin binds or interacts with the bilayer surface is the same as Tien (11) arrived at measuring photoelectric effects on planar bimolecular lipid membranes.

Using an aqueous system and chlorophyllin it has been possible for the first time to differentiate the effect of several components of the polar head group on protecting the

photosynthetic pigment from photooxidation. Some important conclusions on the photooxidation of chlorophyllin can be drawn:

1. Choline protects photooxidation; ethanolamine enhances destruction.
2. Phosphorylcholine protects photooxidation; phosphorylethanolamine enhances destruction.
3. Glycerol has no effect on degradation.
4. Phosphate at pH 7.0 has little effect at low concentration (10 mM) but increases degradation at very high levels (100 mM). Phosphate at pH 11.0 almost totally inhibits degradation.
5. Degradation is enhanced by availability of protons (low pH or ethanolamine).
6. Protection is enhanced by scarcity of protons (high pH or choline).

From the above experiments as well as those of Stillwell and Tien (10) and Van Hasselt et al. (4), it can be concluded that phosphatidylcholine can protect chlorophyll from photooxidation in model systems. Although it remains to be proven that the phospholipid can serve a similar role in vivo, it is a fact that phosphatidylcholine is the second most prevalent phospholipid in thylakoids (16) and its role there has yet to be defined.

It is now believed that chlorophyll is found anchored by its phytol chain in the lipid bilayer of membranes (12-14). The porphyrin head is found at the polar head group region where it sits tilted at a 45° to 54° angle to the rest of the phospholipid molecules (14). With this topography it is reasonable to expect an interaction between the phospholipid and chlorophyll heads. Phospholipids with available protons (such as phosphatidylethanolamine) would enhance degradation and so would be expected to be absent or sequestered away from chlorophyll while protecting phospholipids such as phosphatidylcholine would be expected to be in the immediate vicinity of chlorophyll.

At the thylakoid surface choline may be providing chlorophyll with a degree of protection from the effects of light and oxygen.

REFERENCES:

1. Krasnovsky, A. A. (1960) Ann. Rev. Plant Physiol. 11, 363-410.
2. Goedheer, J. C. (1958) Biochim. Biophys. Acta 27, 478-490.
3. Jen, J. J. and MacKinney, G. (1970) Photochem. Photobiol. 11, 297-302.

4. Van Hasselt, P. R., de Kok, L. J. and Kuiper, P. J. C. (1979) Physiol. Plant, 45, 475-479.
5. Stanier, R. Y. (1959) Brookhaven Symp. Biol. No. 11, 47-53.
6. Cohen-Bazire, G. and Stanier, R. Y. (1958) Nature 181, 250-252.
7. Griffiths, M., Sistrom, W. R., Cohen-Bazire, G. and Stanier, R. Y. (1955) Nature 176, 1211-1214.
8. Zinsou, C. and Costes, C. (1973) Physiol. Veg. 11, 191-206.
9. Krinsky, N. I. (1968) in "Photophysiology" vol. 3 (A. C. Giese, ed.) pp. 123-195, Academic Press, New York.
10. Stillwell, W. and Tien, H. T. (1977) Biochem. Biophys. Res. Comm. 76, 232-238.
11. Huebner, J. S. and Tien, H. T. (1973) Bioenergetics 4, 469-478.
12. Weller, H. and Tien, H. T. (1973) Biochim. Biophys. Acta 325, 433-440.
13. Oettmeier, W., Norris, J. R. and Katz, J. J. (1976) Biochem. Biophys. Res. Comm. 71, 445-451.
14. Luisetti, J., Mohwald, H. and Galla, H. J. (1977) Biochem. Biophys. Res. Comm. 78, 754-760.
15. Bangham, A. D. (1968) Progr. Biophy. Mol. Biol. (J. A. V. Butler and D. Noble, eds.) 18, 29-95, Pergamon Press, New York.
16. Wintermans, J. F. G. M. (1960) Biochim. Biophys. Acta 44, 49-54.