# Structural and functional changes associated with heat-induced phase-separations of non-bilayer lipids in chloroplast thylakoid membranes

Kleoniki Gounaris[+], Anthony P.R. Brain[†], Peter J. Quinn and W. Patrick Williams*

*Departments of [+]Biochemistry, *Biophysics* and [†]Electron Microscope Unit, Chelsea College, University of London, Manresa Road, London SW3 6LX, England*

Received 4 January 1983

The effect of heating isolated bean chloroplasts on the structure of their thylakoid membranes has been examined by freeze-fracture electron microscopy. A normal morphology of the membranes in which stacked grana can be observed is preserved up to 35°C. Incubation at 35–45°C causes complete destacking of the grana but no alteration in the distribution of the membrane-associated particles between the exoplasmic and protoplasmic fracture faces. Heating above 45°C causes phase-separation of non-bilayer lipids into aggregates of cylindrical inverted micelles. Bleaching experiments show that destacking is associated with disruption of chlorophyll–protein complexes of both photosystems I and II. The rate of electron transport through the photosystems is also perturbed. These results are discussed in terms of the role of non-bilayer lipids in packaging the membrane proteins.

*Chloroplast, heat stress* *Membrane lipid, hexagonal phase*
*Galactolipid* *Protein–lipid interaction*

## 1. INTRODUCTION

The occurrence of non-bilayer lipid structures in aqueous dispersions of lipid mixtures in which one, or more, of the components tends to form inverted hexagonal (Hex II) structures when dispersed alone in water is now well established [1–9]. Given that many biological membranes contain high proportions of non-bilayer forming lipids similar structures might be expected to be a common feature of such membranes. The thylakoid membranes of chloroplasts are particularly rich in these lipids; the non-bilayer-forming lipid monogalactosyldiacylglycerol accounting for ~50% of the membrane lipid fractions [10]. Freeze-fracture studies [11] performed on aqueous dispersions of polar lipid extracts of these membranes have revealed a wide range of non-bilayer structures, including spherical and cylindrical inverted micelles sandwiched within lipid bilayers and quasi-crystalline micellar arrays. However, under normal conditions, no corresponding structures are observed in the thylakoids themselves [12].

Studies of binary mixtures of mono- and digalactosyldiacylglycerol in aqueous media [9] suggested that increasing temperature favours the formation of non-lamellar structures whereas low temperature causes the mixture to assume a completely lamellar phase. We have examined the effect of thermal stress on chloroplast membranes to determine the effect of a shift in equilibrium towards creation of non-lamellar lipid structures within the intact membrane. We show that non-bilayer structures of the type seen in lipid dispersions can be induced to form in chloroplast thylakoid membranes by mild heat treatment. Their formation is accompanied by major changes in both chloroplast organisation and function. It is argued that the non-bilayer lipids play a major structural role in the chloroplast associated with the packaging of pigment–protein complexes in the thylakoid membrane.

## 2. EXPERIMENTAL

Chloroplasts were isolated from fresh leaf tissue

*Published by Elsevier Biomedical Press*
00145793/83/0000–0000/$3.00 

of 4–5 weeks post-emergent broad beans (*Vicia faba* cv. Aquadulce) by the method in [13]. Following incubation at a designated temperature, the samples were equilibrated at 25°C, thermally quenched by immersing in a slurry of $N_2$ and fractured at −115°C in a Polaron freeze–fracture device. Comparison with samples thermally quenched from their incubation temperature gave essentially the same results. Replicas were cleaned with bleach, washed, dried and examined in a Philips EM 301 electron microscope.

Photosystem II (PSII)-mediated electron transport was monitored by measurements of the reduction of dichlorophenol–indophenol (DCPIP) as in [14] in the presence or absence of 0.3 mM diphenylcarbazide as a substitute electron donor for $H_2O$. PSI-mediated electron transport was measured by recording the uptake of oxygen associated with electron flow from reduced DCPIP to methyl viologen (MV) in the presence of 6 $\mu$M dichlorophenyldimethylurea to block PSII activity. Full-chain electron transport was determined as the rate of oxygen uptake associated with electron transport from $H_2$ to MV as in [15]. Chloroplasts were uncoupled with 5 mM $NH_4Cl$. All assays were performed at 25°C using either a Cecil CE272 spectrophotometer or a Hansatech (UK) oxygen electrode assembly. Absorption spectra were recorded using a Perkin Elmer 124 Double Beam Spectrophotometer fitted with an integrating sphere.

## 3. RESULTS

The structural consequences of thermal stress in suspensions of broad bean chloroplasts were examined using freeze–fracture electron microscopic techniques. Typical electron micrographs showing some of the main structural features of broad bean chloroplasts that have been incubated at up to 35°C, 35–45°C and >45°C for 5 min are shown in fig.1. Chloroplasts incubated at ≤35°C (fig.1a) show no discernible changes in morphology with respect to non-heated controls. Grana stacking and the usual distribution of intramembranous particles can be clearly observed. Larger (17 nm diam.) particles, mainly located in the stacked granal membranes, are seen in the exoplasmic fracture-faces and smaller (11 nm diam.) particles are distributed fairly evenly throughout the protoplasmic faces of the stacked and unstacked regions. Samples heated to 40°C show a very different organisation (fig.1b,c). No grana stacks are seen and the thylakoids assume a concentric arrangement with relatively few attachment sites between membrane surfaces. The distribution of 17 nm and 11 nm particles between the exoplasmic and protoplasmic fracture-faces, however, is retained and the fracture-plane tends to expose each membrane fracture-face alternately indicating that the membranes have become destacked.

Increasing the incubation temperature to >45°C leads to an extensive phase separation of non-bilayer lipids (fig.1d) involving the formation of three-dimensional aggregates of cylindrical inverted micelles. The individual micelles are 8–10 nm diam. and the aggregates, which usually take the form of closed whorls, are typically 50–200 nm across. They closely resemble structures observed in freeze–fracture replicas prepared from dispersions of total polar lipid extracts of chloroplasts [11] and negatively stained dispersions of mixtures of monogalactosyl and digalactosyldiacylglycerols [7]. Aggregates of spherical inverted micelles of the type seen in such dispersions were not, however, observed in the native membrane preparations. Non-bilayer structures were not found in replicas prepared from chloroplasts incubated at ≤45°C. Following a 5 min incubation at 50°C, however, 75% of the chloroplasts contained non-bilayer structures indicating that a major rearrangement of the chloroplast lipids occurs over 45–50°C. The thermal destacking and phase separation processes were both irreversible.

Functional changes associated with heat-induced structural changes were investigated by measuring rates of electron transport in chloroplast thylakoid membranes after incubation at elevated temperatures. The effect of heat treatment on rates of PSI- and PSII-mediated electron transport are shown in fig.2. In agreement with [16–22], we find that such treatment increases the rate of PSI-mediated electron transport ($DCPIPH_2 \longrightarrow MV$) and decreases the rate of PSII-mediated transport ($H_2O \longrightarrow DCPIP$). Inhibition of PSII activity can be partially restored by use of diphenylcarbazide, an electron donor in place of water [16]. However, even in the presence of diphenylcarbazide, PSII activity is lost if the samples are heated above 44–45°C. Measurements of full-chain electron

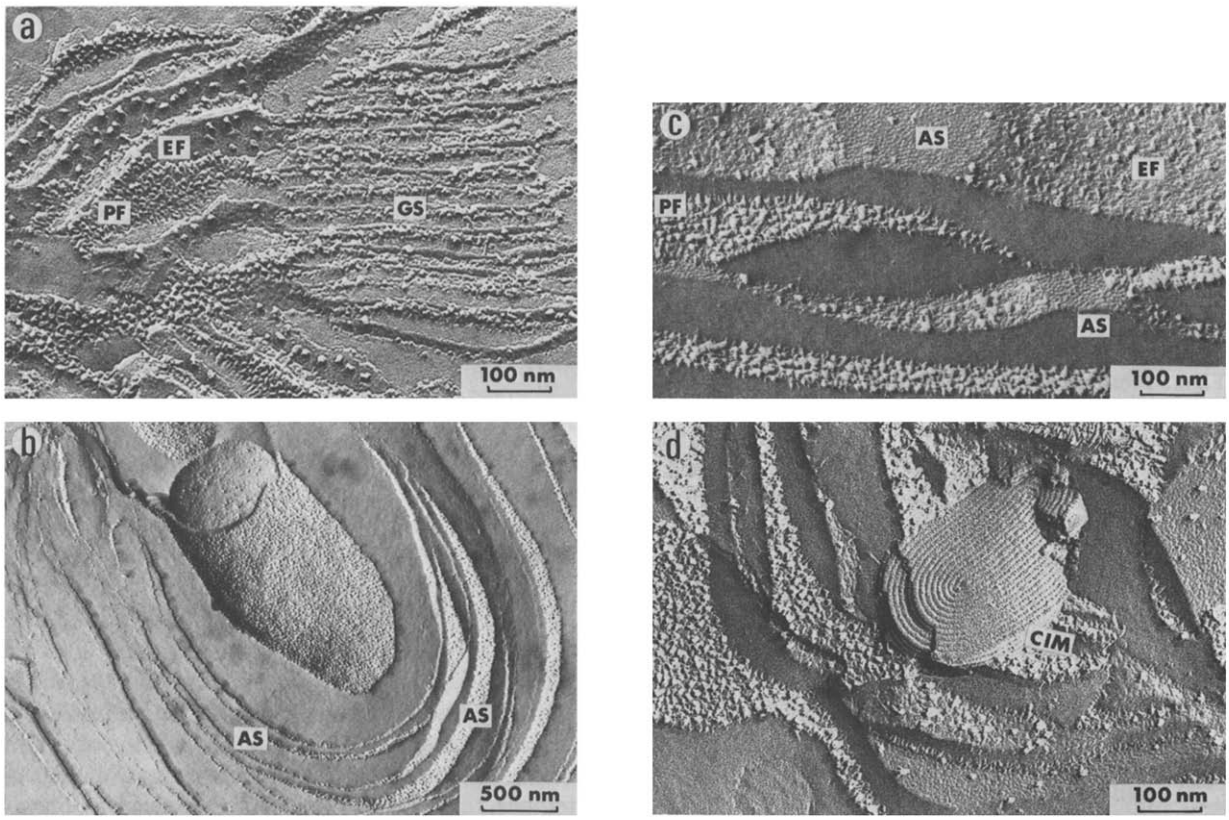


Fig.1. Electron micrographs of freeze–fracture replicas of chloroplasts incubated for 5 min at different temperatures prior to thermal quenching. Samples incubated at 35°C (a) show extensive grana stacking (GS) and the usual distribution of intramembranous particles between the exoplasmic (EF) and protoplasmic (PF) fracture faces of the membranes. Chloroplasts at 40°C (b) show extensive destacking of the thylakoid membrane and a few attachment sites (AS) remaining between membrane surfaces. At higher magnification (c) the EF faces of the membranes at these attachment sites are seen to lack the usual ~17 nm diam. particles, the PF faces (not shown), in contrast, contain many 9–11 nm diam. particles. Chloroplasts incubated at 50°C (d) reveal the formation of large aggregates of cylindrical inverted micelles.

transport ($H_2O \longrightarrow MV$) under coupled and uncoupled conditions (fig.2) confirm earlier reports of heat-dependent uncoupling [17–22]. All these changes in activity, it must be emphasized, appear to take place over the same temperature range as the destacking process described above, suggesting the existence of a direct link between the structural and functional changes.

Spectral studies of heat-treated chloroplast suspensions have indicated that the chlorophyll–protein complexes of PSI are destabilized at high temperatures. Exposure of preheated chloroplasts to strong white light, for example, leads to a substantial bleaching in both the blue and the red regions of the spectrum (fig.3). The difference spectrum obtained by subtraction of the absorption spectrum of the illuminated sample from that of the non-illuminated control shows maxima at about 440, 490 and 680 nm. The loss of absorption at around 680 nm appears to reflect a preferential bleaching of P700/chlorophyll *a* protein (the major chlorophyll–protein complex of PSI) which absorbs maximally in this wavelength region [23]. Chlorophyll *b* absorbing maximally around 650 nm, does not appear to be bleached suggesting that the chlorophyll *a*/chlorophyll *b* light-harvesting protein (LHCP) associated with PSII is not photolabile under these conditions. The changes observed in the blue region of the spectrum are more complex probably reflecting the for-

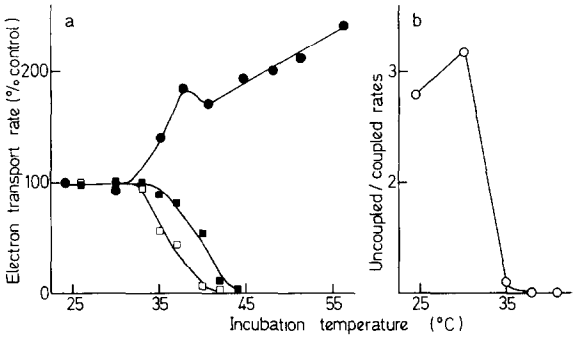


Fig.2. Plots showing the effect of 5 min incubation at different temperatures on the rates of (a) uncoupled PSII-mediated electron transport in the presence (■) and absence (□) of diphenylcarbazide and PSI mediated electron transport (●). (b) The relative rates of coupled and uncoupled full-chain electron transport.

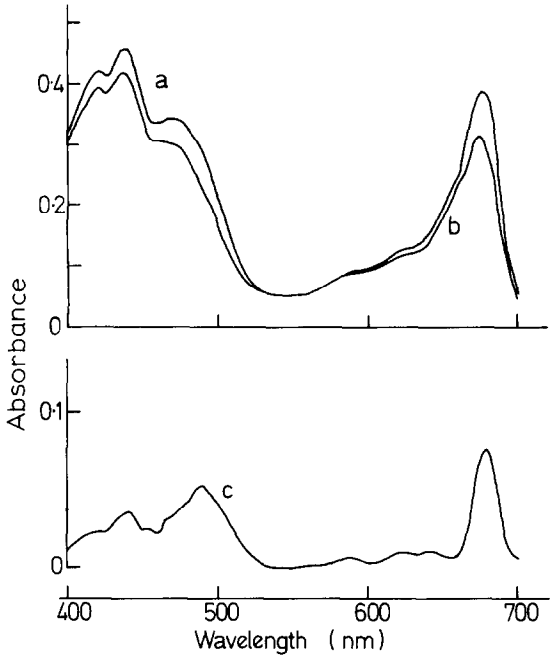


Fig.3. Absorption of: (a) broad bean chloroplasts incubated at 42°C for 5 min prior to measurements; (b) the same sample after 15 min illumination with white-light (intensity 1.1 $W.m^{-2}$) at 25°C; (c) difference spectrum obtained from (b)–(a).

mation of chlorophyll degradation products absorbing at these wavelengths. Measurements of chlorophyll bleaching as a function of incubation temperature are presented in fig.4. They show that

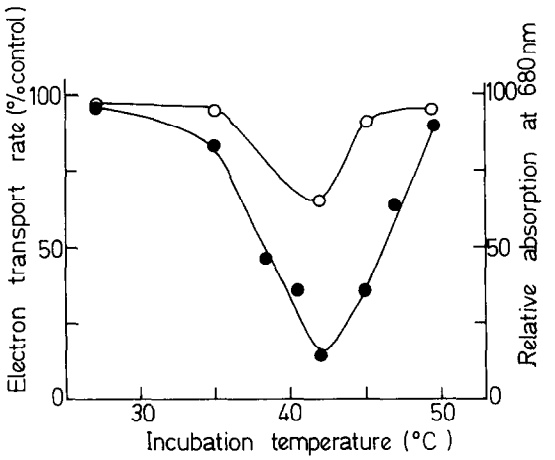


Fig.4. Plots showing the effect of 15 min white-light illumination on absorption at 680 nm and rates of PSI-mediated electron transport ($DCPIPH_2 \rightarrow MV$) of chloroplast samples incubated for 5 min at different temperatures. Heated but non-illuminated samples were used as controls.

the threshold temperature for chlorophyll bleaching is 35–40°C. It is noteworthy that the lability of these pigments is reduced at >45°C; the temperature range in which extensive phase-separation of the non-bilayer lipids is observed. Parallel measurements of PSI-mediated electron transport show similar effects confirming that the bleached pigments are indeed associated with PSI.

## 4. DISCUSSION

In [24] we suggested that the presence of non-bilayer lipids is required to package the light-harvesting units of PSI and PSII (equivalent $M_r \sim 1000–5000 \times 10^3$) into the chloroplast membranes. Furthermore, the maintenance of an appropriate balance between bilayer and non-bilayer forming lipids is supposed to be a major constraint on the temperature lability of the thylakoid membrane [11]. Studies on model systems [2,9] indicate that non-bilayer forming lipids have an increased tendency to phase-separate at higher temperatures. In the case of the thylakoid membrane, this tendency appears to manifest itself first in a destabilisation of the chlorophyll–protein complexes of the two photosystems and then, at slightly higher temperatures, in a phase-separation of the non-bilayer forming lipids. The loss of func-

tional activities associated with these structural changes is clearly evident here. It was originally thought that loss of the ability of chloroplasts to evolve $O_2$ following heat treatment was due to a breakdown of electron transport between the water-splitting enzyme and the reaction centre of PSII [16] and specifically the disruption of a protein on the donor side of PSII [25]. Other studies based on fluorescence measurements [26,27] have tended to favour the idea that heat-treatment disrupts interactions between different components of the PSII light-harvesting system and the PSII reaction centres. One of the major components of this light-harvesting system is LHCP which, in addition to its light-harvesting role, is believed to play an important role in grana stacking [29]. The fact that loss of PSII-mediated electron transport is accompanied by simultaneous destacking of the grana membranes argues strongly that heat-treatment results in major changes in the local environment of this protein.

The involvement of non-bilayer lipid–protein interactions may explain why, despite their spatial separation, PSI and PSII show very similar threshold temperatures for their changes in electron-transport activity (fig.2,4). It also accounts for earlier observations that these threshold temperatures can be readily modified by alterations in the growth temperature of the plants from which the chloroplasts are isolated [19,20,29]. Such changes, whilst unlikely to affect the stability of the pigment–protein complexes themselves, are known to lead to alterations in lipid composition [30] and/or lipid–protein ratios [31].

We have shown that, in addition to thermal stress, non-bilayer structures can be induced to form in chloroplast membranes by freeze–thawing and ageing (unpublished). Other workers have observed, but not recognised, similar structures in thylakoids washed with guanidinium hydrochloride [32] or exposed to high $[Mg^{2+}]$ [33]. Non-bilayer structures have also been found in the membranes of partially dehydrated visual rod outer segments [34,35], sarcoplasmic reticulum [36] and liver mitochondria treated with high $[Mn^{2+}]$ [37]. It would appear that any treatment tending to destabilise lipid–protein interactions is likely to induce phase-separations in membranes rich in non-bilayer lipids. If this is indeed the case, a better understanding of the precise role of such lipids within biological membranes must be a matter of prime importance.

## ACKNOWLEDGEMENTS

This work was aided by a grant from the Agricultural Research Council. Equipment was purchased by funds provided by the Royal Society and the Central Research Fund of London University.

## REFERENCES

[1] Verkleij, A.J., Mombers, C., Leunissen-Bijvett, J. and Ververgaert, P.H.J.T. (1979) Nature 279, 162–163.
[2] De Kruijff, B., Verkleij, A.J., Van Echteld, C.J.A., Gerritsen, W.J., Mombers, C., Noordam, P.C. and De Grier, J. (1979) Biochim. Biophys. Acta 555, 200–209.
[3] Verkleij, A.J., Mombers, C., Gerritsen, W.J., Leunissen-Bijvett, L. and Cullis, P.R. (1979) Biochim. Biophys. Acta 555, 358–361.
[4] Miller, R.G. (1980) Nature 287, 166–167.
[5] Hui, S.W., Stewart, T.P., Yeagle, P.L. and Albert, A.D. (1981) Arch. Biochem. Biophys. 207, 227–240.
[6] Van Venetie, R. and Verkleij, A.J. (1981) Biochim. Biophys. Acta 546, 262–269.
[7] Sen, A., Williams, W.P., Brain, A.P.R., Dickens, M.J. and Quinn, P.J. (1981) Nature 293, 488–490.
[8] Sen, A., Williams, W.P., Brain, A.P.R. and Quinn, P.J. (1982) Biochim. Biophys. Acta 685, 297–306.
[9] Sen, A., Brain, A.P.R., Quinn, P.J. and Williams, W.P. (1982) Biochim. Biophys. Acta 686, 215–224.
[10] Nishihara, M., Yokota, K. and Kito, M. (1980) Biochim. Biophys. Acta 617, 12–19.
[11] Gounaris, K., Sen, A., Brain, A.P.R., Quinn, P.J. and Williams, W.P. (1983) Biochim. Biophys. Acta, in press.
[12] Quinn, P.J., Gounaris, K., Sen, A. and Williams, W.P. (1982) in: Biochemistry and Metabolism of Lipids (Wintermans, J.F.G.M. and Kuiper, P.J.C. eds) pp.327–330, Elsevier Biomedical, Amsterdam, New York.
[13] Stokes, D.M. and Walker, D.A. (1971) Plant Physiol. 48, 163–165.
[14] Vernon, L.P. and Shaw, E.R. (1969) Plant Physiol. 44, 1645–1649.
[15] Restall, C.J., Williams, W.P., Percival, M.P., Quinn, P.J. and Chapman, D. (1979) Biochim. Biophys. Acta 555, 119–130.

[16] Yamishita, T. and Butler, W.L. (1968) Plant Physiol. 43, 2037–2040.
[17] Emmet, J.M. and Walker, D.A. (1969) Biochim. Biophys. Acta 180, 424–425.
[18] Krause, G.H. and Santarius, K.A. (1975) Planta 127, 285–299.
[19] Pearcy, R.W., Berry, J.A. and Fork, D.C. (1977) Plant Physiol. 59, 873–878.
[20] Armond, P.A., Schreiber, U. and Bjorkman, O. (1978) Plant Physiol. 61, 411–415.
[21] Santarius, K.A. (1980) Plant Physiol. 49, 1–6.
[22] Stidham, M.A., Uribe, E.G. and Williams, G.J. (1982) Plant Physiol. 69.
[23] Thornber, J.P. (1975) Ann. Rev. Plant Physiol. 26, 127–158.
[24] Williams, W.P., Sen, A. and Quinn, P.J. (1982) Biochem. Soc. Trans. 10, 335–338.
[25] Cramer, W.A., Whitmarsh, J. and Low, P.S. (1981) Biochemistry 20, 157–162.
[26] Schreiber, U. and Berry, J.A. (1977) Planta 136, 233–238.
[27] Schreiber, U. and Armond, P.A. (1978) Biochim. Biophys. Acta 502, 138–151.
[28] Ryrie, I.J., Anderson, J.M. and Goodchild, D.J. (1980) Eur. J. Biochem. 107, 345–354.
[29] Berry, J. and Bjorkman, O. (1980) Ann. Rev. Plant Physiol. 31, 491–543.
[30] Quinn, P.J. and Williams, W.P. (1978) Prog. Biophys. Mol. Biol. 34, 109–173.
[31] Chapman, D.J., Milner, P.A., Ford, R.C. and Barber, J. (1982) in: Biochemistry and Metabolism of Lipids (Wintermans, J.F.G.M. and Kuiper, P.J.C. eds) pp.363–368, Elsevier Biomedical, Amsterdam, New York.
[32] Machold, O., Simpson, D.J. and Hoyer-Hansen, G. (1977) Carlsberg Res. Commun. 42, 499–516.
[33] Carter, D.P. and Staehelin, L.A. (1980) Arch. Biochem. Biophys. 200, 374–386.
[34] Corless, J.M. and Costello, M.J. (1982) Exp. Eye Res. 32, 217–228.
[35] Gruner, S.M., Rothschild, K.J. and Clark, N.A. (1982) Biophys. J. 39, 241–245.
[36] Crowe, L.M. and Crowe, J.H. (1982) Arch. Biochem. Biophys. 217, 582–587.
[37] VanVenetie, R. and Verkleij, A.J. (1982) Biochim. Biophys. Acta 692, 397–405.