Biochimica et Biophysica Acta 849 (1986) 325–336
Elsevier

BBA 42001

Homogeneous catalytic hydrogenation of lipids in the photosynthetic membrane: effects on membrane structure and photosynthetic activity

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(Received October 18th, 1985)

Key words: Chloroplast membrane; Membrane fluidity; Photophosphorylation; Electron transport; Lipid hydrogenation; (Pea chloroplast)

We have carried out a series of experiments in which the lipid composition of the photosynthetic membrane has been altered by the homogeneous catalytic hydrogenation of the unsaturated fatty acid residues of membrane lipids. The modified membrane was investigated by electron microscopy, electron-spin resonance and fluorescence polarization methods. Alteration in the functional characteristics of the hydrogenated membrane was monitored by the measurement of photophosphorylation and electron-transport activities. The following results were found. (a) Saturation of 10% of the fatty acyl double bonds induced a definite decrease in the dimension of both thylakoids and loculi. Microdensitometry showed that these structural changes arose from a thickening of the single membranes with a simultaneous decrease in the spacing between membranes. These changes might be accounted for by the alignment of the hydrocarbon chains of saturated lipids and the increased hydrophobicity of the membranes. (b) The orientational pattern of chlorophyll-*a* molecules was not altered by saturating up to 50% of fatty acyl double bonds in membrane lipids, indicating that the energy-transfer processes amongst the chlorophyll molecules remained functional after hydrogenation. (c) Saturation of double bonds of lipids inhibited whole electron transport prior to the inhibition of Photosystem II and Photosystem I activity, which may suggest that the unsaturation level of fatty acids plays a crucial role by ensuring the lateral mobility of plastoquinone between Photosystem II and Photosystem I.

Introduction

Chloroplast thylakoid is an example of a highly specialized, vectorially oriented membrane which provides an ideal system for studying the relationship between membrane structure and function. Functional characteristics of these membranes include the light-driven reactions which are defined by a highly ordered, structural arrangement. Photon-absorbing pigments and the majority of redox components involved in subsequent electron transport from water to appropriate electron-accepting molecules are incorporated into four macromolecular protein complexes: the light-harvesting chlorophyll *a/b* protein (LHCP); Photosystem II (PS II); Photosystem I (PS I) and the cytochrome b_6/f complex [1,2]. These complexes, along with the

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Abbreviations: DCIP, dichlorophenolindophenol; DGDG, digalactosyldiacylglycerol; DQH_2, duroquinol; *F*, fluorescence; pBQ, *p*-benzoquinone; PS, Photosystem; 16-SASL, 16-(4′,4′-dimethyloxazolidine-*N*-oxyl)stearic acid; LHCP, light-harvesting chlorophyll protein; Ph, pheophytin; Mes, 4-morpholineethanesulphonic acid; PMS, phenazine methosulphate; PC, phosphatidylcholine.

ATP synthase complex (CF_0-CF_1) [3], are embedded in the lipid matrix of thylakoid membranes [4]. It is reasonable to expect that the lipid composition of the membrane plays an important role in thylakoid organization by determining the cooperative interaction among the functional complexes. Functional interactions of the complexes depend on several diffusion processes, which probably involve rotational and lateral movements within the membrane and are consequently dependent on the fluidity of the lipid matrix [5,6]. Fluidity characteristics of the lipid bilayer are primarily determined by the length and the degree of saturation of alkyl chains in fatty acids of constituent lipids [7,8]. It is noteworthy that the lipid/protein ratio can influence the fluidity characteristics of the thylakoid membranes as well [9]. In addition to the general fluidity characteristics, fatty acyl residues, in particular, have been implicated in biological function [10].

Chloroplast membranes are characterized by a large proportion of polyunsaturated lipids. The major acyl residue in chloroplast membrane lipids is linolenic acid (18 : 3) which can reach at least 70% of the total fatty acid content of higher plant chloroplast lamellae [11,12]. It has been suggested that polyunsaturated fatty acids are required to maintain the appropriate membrane fluidity over a wide range of environmental temperature and to induce chilling resistance and cold hardiness [6]. Since the functional significance of chloroplast lipid is not yet fully clarified it further requires detailed investigations. A potential method for testing the possible role of unsaturated lipids is investigating the effect of selective modification of the constituent lipids within the membrane.

Homogeneous catalytic hydrogenation was first developed by Chapman and Quinn [13], who applied it in both model and living systems [13,14]. This technique allows the saturation of fatty acyl double bonds in situ retaining the pre-existing architecture of the membrane [14]. However, the catalyst proved water-insoluble and thus remains associated with the membrane at the end of the reaction [14]. Recently, we have introduced a homogeneous catalytic hydrogenation procedure by applying a water-soluble catalyst, a Pd(II) sulphonated alizarine complex ($Pd(QS)_2$) [15]. This catalyst aids in overcoming the disadvantage of Chapman and Quinn's technique, since it can be simply removed by washing at the completion of the reaction. The effect of catalytic hydrogenation on fatty acid patterns of chloroplast thylakoids is described in our previous articles [15,16].

It is shown that within 4 h of hydrogenation about 60% of the double bonds of fatty acids are saturated. The analysis of fatty acid patterns demonstrates that our hydrogenation procedure is markedly selective in the saturation of polyenoic acids, such as C18 : 3, by enhancing mainly C18 : 1 and C18 : 0 [16]. Simultaneously, the order parameter of spin-labelled fatty acid is increased, indicating extensive structural changes within the chloroplast thylakoids [16]. In order to obtain more information concerning the nature of these structural changes and their consequences in thylakoid functions, we carried out a detailed investigation of ultrastructure in relation to various functional characteristics of chloroplasts subjected to hydrogenation.

In this study we report the effects of homogeneous catalytic hydrogenation on the photosynthetic membrane, by applying a water-soluble $Pd(QS)_2$ catalyst. We have analyzed the membranes, produced following this procedure, in terms of their biochemical, structural and biophysical characteristics. Our data demonstrates that the modified thylakoids differ from the untreated ones in a number of fundamental ways. These observations suggest that the saturation level of fatty acids of lipids plays a crucial role in determining the structural organization and functional characteristics of photosynthetic membranes.

Materials and Methods

Plant material. Peas (*Pisum sativum* L.) were germinated and grown under greenhouse conditions for 14 days, then transferred to a Mytron KLTK 1250 phytotrone (VEB Maschinenfabrik Nema, Netzschkau, G.D.R.) for 7 days, where seedlings were grown under 22°C/18°C, day/night temperatures. Leaves were harvested after 21 days of growth.

Chloroplast preparation. Leaves were homogenized at 0°C in a medium containing 0.4 M D-sorbitol/10 mM NaCl/5 mM $MgCl_2$/2 mM

EDTA/1 mM $MnCl_2$/2 mM ascorbate/0.4% bovine serum albumin/50 mM Mes (pH 6.5) [17]. The brei was filtered through four layers of nylon cloth, while chloroplasts were sedimented by centrifugation for 5 min at 3000 × *g*. The chloroplasts were washed once in the isolation medium, then resuspended in a medium containing 0.4 M D-sorbitol/10 mM NaCl/2 mM $MgCl_2$/2 mM EDTA/1 mM $MnCl_2$/0.4% bovine serum albumin/50 mM Hepes (pH 7.5) to give a chlorophyll concentration of 10 μg/ml.

Hydrogenation procedure. The catalyst used in the experiments was $Pd(QS)_2$, the sulphonated alizarine complex of Pd(II), and prepared as described in Ref. 18. 30 ml of chloroplast suspension contained in high-pressure glass vessels was transferred to a thermostated (25°C) water bath. The reactors, connected to vacuum-gas manifolds, were evacuated and subsequently filled with hydrogen at a pressure of 0.1 MPa. The catalyst solution was injected into the suspension (chlorophyll/catalyst ratio, 0.125) through a silicone rubber septum. The hydrogen pressure was then increased to 0.3 MPa, and the reaction tubes were rotated at 45 rpm for the desired reaction time. At the end of incubation, the pressure was slowly released over a period of 5 min. To remove the catalyst, samples were washed 3 times with fresh resuspension medium. The removal of the catalyst was controlled by absorption spectroscopy. After the third washing no detectable amount of catalyst could be observed in the absorption spectra of the chloroplast suspension [16]. Chloroplasts were sedimented by centrifugation for 5 min at 3000 × *g*. In control experiments, chloroplasts were treated with catalyst under N_2 pressure [16]. Extraction of lipids and the analysis of fatty acids were performed as in Ref. 19.

Chlorophyll determination. Before and after hydrogenation aliquots of the chloroplast suspension were extracted by 80% acetone and the pigment extract was measured spectrophotometrically as described by Arnon [20].

Electron microscopy. Chloroplast pellets were fixed, dehydrated, embedded, sectioned and stained using conventional methods. Membrane thickness and dimensions of loculi were recorded by tracing the electron-micrograph negatives on a microdensitometer coupled with an *XY* recorder. The microdensitometer operated at a slit corresponding to a microbeam of 40–50 μm on the negative. The density of the surrounding stroma was taken as the baseline. Membrane thickness is given as the curve width at half maximal value of the densitometric peak. The width of the loculi was calculated from the peak-to-peak distance of the densitometric curves and the distance corresponding to the thickness of the membranes [21].

Fluorescence polarization measurements. Chloroplast membrane fragments were embedded in polyacrylamide gel [22] and aligned by uniaxial squeezing [23]. The squeezing parameter was $m = L_{zo}/L_z = 1.9$, where L_{zo} and L_z match the thickness of the sample before and after squeezing. The fluorescence measurements were carried out at −196°C. Samples were excited with non-polarized light at the wavelengths of the blue spectral lines of an HBO 200 high pressure Hg arc lamp. Fluorescence was observed in the direction perpendicular to that of the squeezing and excitation, and recorded with polaroid sheets in positions transmitting vertical (F_v) and horizontal (F_h) polarized light. In oriented samples the fluorescence polarization ratio spectra, determined by F_v/F_h, corresponded to a fluorescence polarization value of $F_{\parallel}/F_{\perp}$; $F_{\parallel}$ and $F_{\perp}$ were the fluorescence-intensity components emitted parallel and perpendicular to the plane of the aligned membranes, respectively.

Electron-spin resonance studies. 200 μl aliquots containing 0.4 mg chlorophyll were labelled by the addition of 2 μl ethanolic solution of 16-(3′,4′-dimethyloxazolidine-*N*-oxyl) stearic acid, 16-SASL (corresponding to 20 μg label) and vigorously mixed with a Vortex mixer for 5 min. The membrane suspension was transferred into a 100 μl capillary and pelletted down in a bench centrifuge. ESR spectra were recorded with a JEOL JES-PE-1X spectrometer using 100 kHz modulation technique. During measurements the sample capillary was thermostatically controlled in a nitrogen gas flow system. At the depth of C-16 carbon atoms, no significant signal reduction was observed and, therefore, the rotational correlation time was calculated from the uncorrected peak-to-peak amplitudes and widths as described by Schreier et al. [24].

Measurement of photosynthetic activity. The rate of photosynthetic oxygen evolution and uptake was measured by using a Clark-type electrode (Rank Broths, Cambridge, U.K.) in a temperature-controlled cuvette under saturating white light [25]. The assay medium contained 0.1 M sorbitol, 4 mM $MgCl_2$, 20 mM NaCl, 10 mM K_2HPO_4, 2 mM EDTA, 50 mM Hepes (pH 7.5) and chloroplasts equivalent to 50 μg chlorophyll per sample [17].

Different parts of the electron-transport chain were studied by addition of various electron donors and acceptors: 2 mM $K_3Fe(CN)_6$ or 0.1 mM methyl viologen together with 2 mM NaN_3 were used for whole-chain electron transport. PS II electron transport was measured by using 0.25 mM *p*-benzoquinone. For the PS I reaction, electron transport was monitored by using either 40 μM dichlorophenolindophenol and 2 mM ascorbate or 125 μM duroquinol, depending on the system used. Duroquinone was reduced to duroquinol by $NaBH_4$ according to Izawa [26]. The rate of uncoupled electron flow was measured by adding 10 mM methylamine to the samples.

Measurement of photophosphorylation. The light-induced ATP formation of chloroplasts was estimated by monitoring the bioluminescence of the firefly luciferin/luciferase in a home-made luminometer. 2 ml of the reaction mixture contained 40 μM ADP, 75 μM phosphate buffer (pH 7.2) and chloroplasts equivalent to 0.5 μg chlorophyll/ml. Luciferin/luciferase was obtained from LKB ATP Monitoring Kit (LKB Wallac, Turku, Finland) and dissolved in 10 ml water, 0.2 ml of this solution was added to 1.8 ml of reaction mixture. Measuring cyclic phosphorylation, 50 μM phenazine methosulphate was added to a fresh sample. Experiments were calibrated, individually, using known concentrations of ATP. Samples were placed in a 1 cm cuvette which was illuminated through a horizontal light-guide equipped with 650 W tungsten lamp. Actinic light passed through Calfex C (Balzers Union AG, Liechtenstein), Wratter 29 filters (Kodak, Paris, France) and a Corning CS 2-64 filter transmitting light over 650 nm. The photomultiplier was protected by a Corning CS 4-95 blue filter.

Results

Chloroplast ultrastructure

The structure of untreated and hydrogenated chloroplasts were examined under the electron microscope. In the ultrastructure of chloroplasts subjected to hydrogenation (58% saturation in the double bonds of fatty acids), no dramatic changes could be observed, since their basic structure remained unchanged and grana stacking was preserved (Fig. 1a and b). The only virtual change found was that hydrogenated chloroplasts seemed to be more intensively stained than the untreated control. This suggests that the granal structures become more compact following hydrogenation. To quantitate changes in the granal region, the electron micrographs of samples were subjected to analysis by microdensitometry [21]. Typical microdensitometric tracings of a granum before and after hydrogenation are shown in Fig. 2. The peak represents the well-stained double membranes of the thylakoid (the stacked membranes of two grana vesicles), while the dip corresponds to non-stained, interthylakoidal space or loculus (cf. Figs. 1 and 2). It can be seen that the thickness of thylakoids as well as the dimension of loculi were decreased upon hydrogenation. The numerical evaluation of these changes was obtained from measurements of 150 membranes of 15 micrographs for each treatment (data displayed in Table I). Results clearly show that even 10% saturation of double bonds in thylakoid membrane lipids can induce a 15%

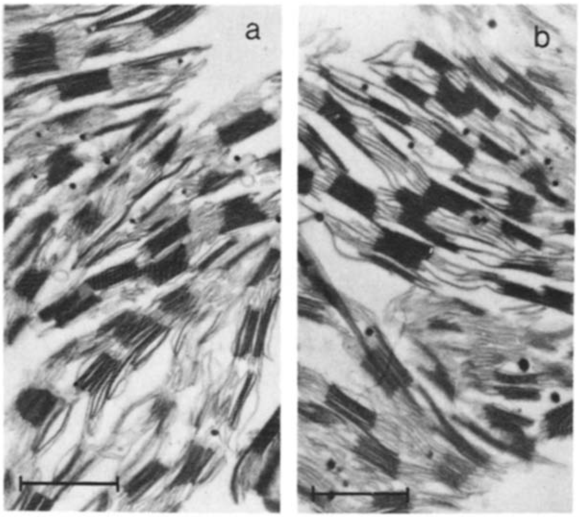

Fig. 1. Ultrastructure of chloroplasts before and after hydrogenation (a) control chloroplasts, (b) chloroplasts subjected to 4 h of hydrogenation. Magnification: ×14000

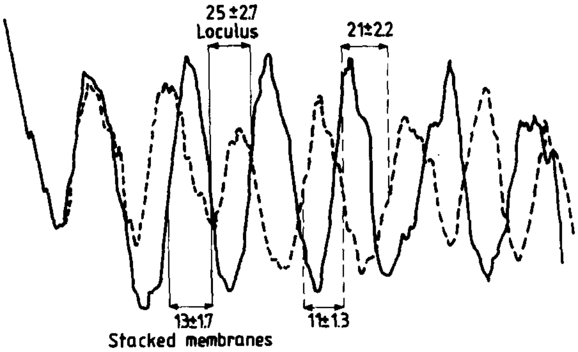

Fig. 2. Microdensitometric traces of thylakoids at grana region of control (———) and hydrogenated (— — —) chloroplasts. In hydrogenated chloroplasts, 48% of fatty acyl double bonds were saturated. Other conditions as in Fig. 1 and Methods.

shrinkage of the stacked membranes and about 25% decrease in the dimension of loculi resulting in a flattening of the thylakoids. Further saturation of double bonds does not alter the dimensions of either the membranes or the loculi. Since most of the in vivo membrane shrinkage may be accounted for by conformational changes within the membranes [21,27], some alteration in the orientation pattern of the pigments may be expected.

Orientation pattern of chlorophyll a in thylakoid membranes

The orientation of chlorophyll *a* molecules was characterized by the low temperature, polarized fluorescence spectra of isolated chloroplasts. The spectrum of control chloroplasts exhibited prominent bands at 685, 695 and 735 nm (Fig. 3A). After saturation of nearly 60% of lipid double bonds, the band at 695 nm in polarized fluorescence spectra disappeared and only peaks at 685 and 735 nm were observable (Fig. 3B). In the polarized fluorescence spectra of both control and hydrogenated chloroplasts, the intensity of $F_{\parallel}$ was much higher than that of $F_{\perp}$. Fluorescence-polarization ratios, calculated from polarized fluorescence spectra, showed a high degree of chlorophyll orientation in both untreated and hydrogenated chloroplasts with fluorescence-polarization values of 1.3–1.9 between 680 and 740 nm (Fig. 4). During hydrogenation, no significant changes could be observed in the fluorescence-polarization values of the different chlorophyll forms up to 50% saturation of lipid double bonds. When about 60% of the fatty acyl double bonds were saturated, the dip at 695 nm disappeared and the fluorescence-polarization values of the longer-wavelength chlorophyll forms were elevated.

The inset of Fig. 4 shows that normal low-temperature fluorescence spectra of isolated chloroplasts exhibit peaks at 685, 695 and 735 nm. Hydrogenation did not change the spectra indicating that the individual chlorophyll forms, themselves, were not altered by the catalytic procedure. This is in agreement with our previous observation that our in situ hydrogenation procedure did not saturate conjugated double bonds of the photosynthetic pigments such as carotenoids and chlorophylls (Szalontai, B., unpublished observations). We conclude that the light-harvesting and energy-transfer pigment systems of the photosynthetic apparatus were not directly attacked by homogeneous catalytic hydrogenation.

TABLE I

CHANGES IN DIMENSIONS OF STACKED MEMBRANES AND LOCULI IN GRANA OF PEA CHLOROPLASTS AT VARIOUS LEVELS OF HYDROGENATION

Hydrogenation		Stacked membranes			Loculi		
average number of double bonds per lipid molecule	percentage of saturation	nm ± S.D.	percentage of control	changes in percentage	nm ± S.D.	percentage of control	changes in percentage
3.49	0	12.96 ± 1.66	100	0	12.45 ± 2.13	100	0
3.09	11	11.08 ± 1.38	85	−15	8.96 ± 1.23	72	−28
2.65	24	11.50 ± 1.50	89	−11	9.38 ± 2.67	75	−25
2.27	35	10.73 ± 2.16	83	−17	9.46 ± 1.17	76	−24
1.81	48	11.15 ± 1.35	86	−14	9.63 ± 1.68	77	−23

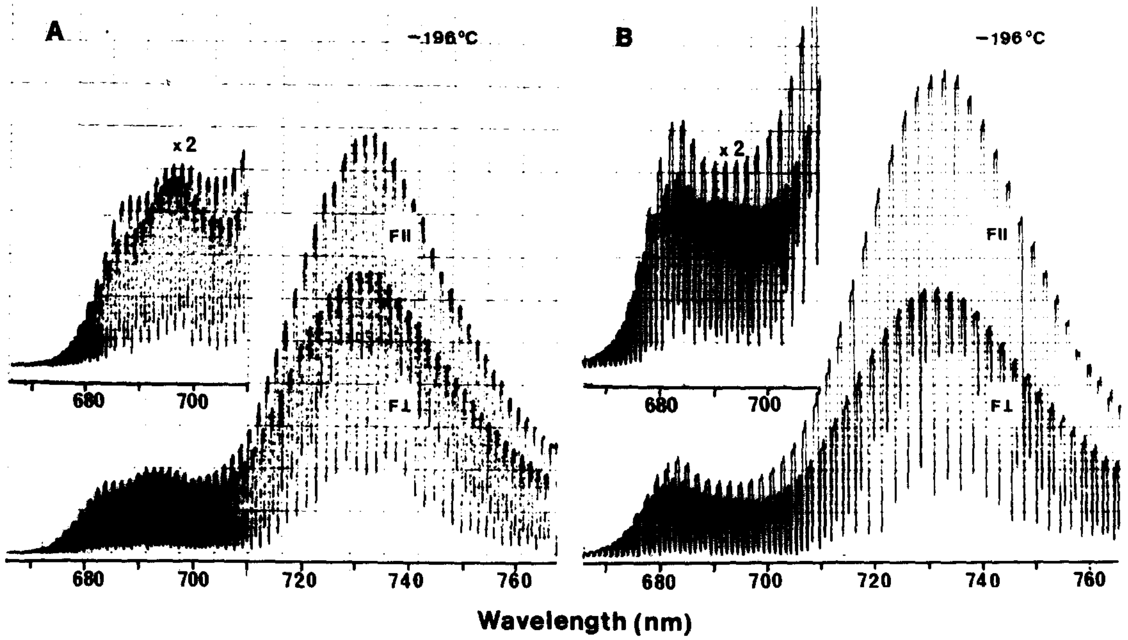

Fig. 3. Polarized fluorescence spectra of control (A) and 58% hydrogenated (B) chloroplasts measured at liquid nitrogen temperature. The insets represent a 2-fold magnification of the short-wavelength part of the spectra. Measuring conditions are given in Materials and Methods.

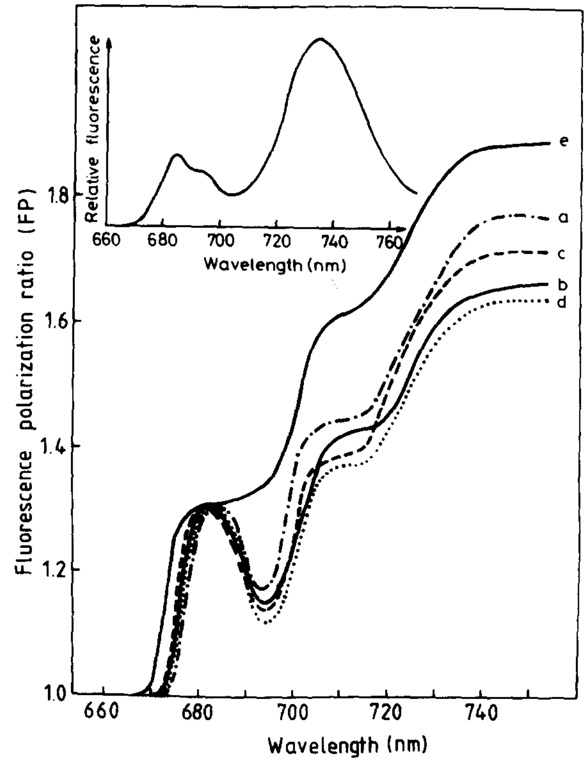

Fig. 4. The fluorescence polarization (FP) spectra of chloroplasts subjected to hydrogenation to various extents: 0% (a), 11% (b), 24% (c), 35% (d) and 48% (e) saturation of fatty acyl double bonds. The inset displays the normal fluorescence spectra of chloroplasts measured at −196°C.

Photosynthetic electron-transport activity

Fig. 5 demonstrates the effects of saturation of membrane lipid double bonds on the photosynthetic electron-transport activity. The entire electron transport measured from $H_2O \rightarrow K_3Fe(CN)_6$, or $H_2O \rightarrow$ methyl viologen, gradually decreased as hydrogenation progressed. PS II electron transport measured from $H_2O \rightarrow pBQ$ was inhibited only when more than 10% of lipid double bonds were saturated and exhibited gradual decrease upon further hydrogenation. PS I activity measured from $DQH_2 \rightarrow$ methyl viologen also showed partial inhibition when 10% double bonds of lipids were saturated, but it was not changed by progressive hydrogenation until about 50% saturation of double bonds. DCIP → methyl viologen reaction, representative of PS I electron flow from cytochrome *f*/plastocyanin to P-430 was only in-

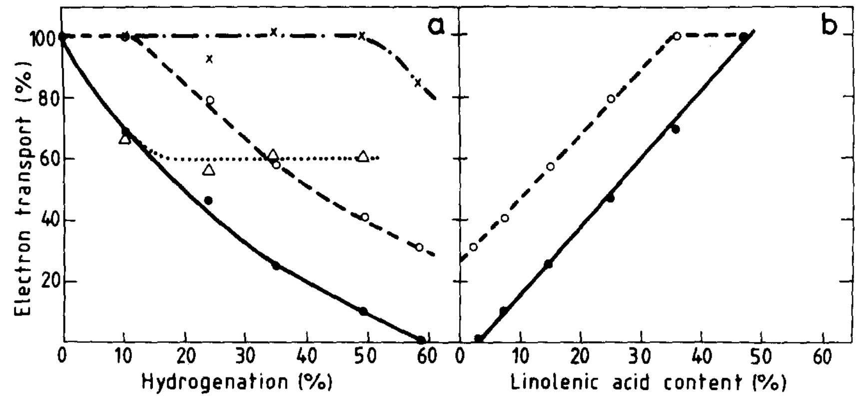

Fig. 5. Effect of hydrogenation on the activity of the different parts of the photosynthetic electron-transport chain. (a) Changes in the electron-transport activity during hydrogenation. (b) Electron-transport activity plotted as the function of linolenic acid content of the membranes varied by hydrogenation. Whole electron transport measured from $H_2O \rightarrow$ methyl viologen (●——●); PS II electron transport from $H_2O \rightarrow$ pBQ, (○------○); PS I activity measured from $DQH_2 \rightarrow$ methyl viologen (△·····△); or from DCIP → methyl viologen (×-·-·-×). Measuring conditions as in Materials and Methods. The uncoupled electron-transport activity in control chloroplasts expressed in μmol O_2 per mg Chl per h are as follows: $H_2O \rightarrow$ methyl viologen, 275; $H_2O \rightarrow$ pBQ, 263; $DQH_2 \rightarrow$ methyl viologen, 268; DCIP → methyl viologen, 298.

hibited by hydrogenation when more than 50% of double bonds were saturated.

Fig. 5b demonstrates that the inhibition of whole electron transport from $H_2O \rightarrow$ methyl viologen shows a linear dependency with the decrease of linolenic acid content of thylakoid membranes, PS II electron transport showed a similar linearity when it was plotted as the function of linolenic acid content of the thylakoid. These observations may indicate that the linolenic acid content of the membrane has an important role in controlling the electron transport between the two photosystems as well as within PS II.

Temperature dependency of the electron-transport activity of both hydrogenated and untreated chloroplasts is shown in Fig. 6. Control chloroplasts exhibited a break-point at around 9°C in the Arrhenius plots of the Hill activity, measured from $H_2O \rightarrow$ methyl viologen in the presence of 10 mM methylamine. Similar discontinuities could be observed when an uncoupler was omitted from the reaction mixture (data not shown). This is in disagreement with earlier observations of Nolan and Smillie [28,29] who obtained discontinuities in the Arrhenius plots of Hill activity only when the chloroplasts were uncoupled. The hydrogenated chloroplasts had a lower rate in the Hill activity than that of the control and, surprisingly, no

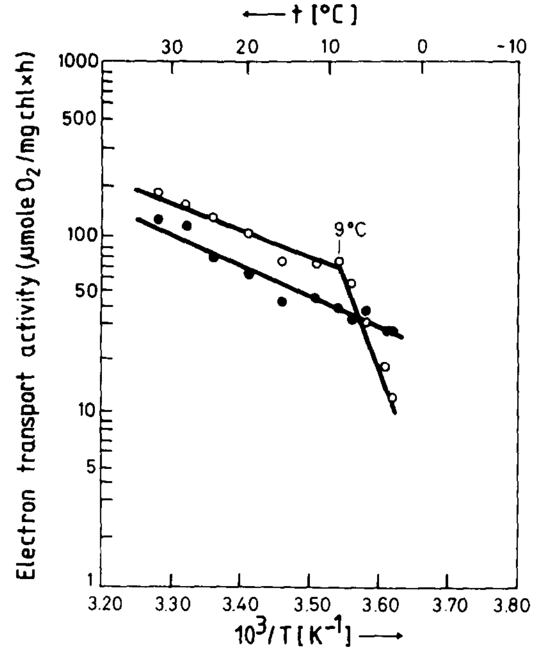

Fig. 6. Arrhenius plots of photosynthetic electron-transport activity in control (○) and partially hydrogenated chloroplasts (●) represent 15% saturation of fatty acyl double bonds. Whole electron transport was measured from $H_2O \rightarrow$ methyl viologen at various temperatures. Other measuring conditions as given in Materials and Methods.

discontinuities could be observed in the temperature range between 0°C and 32°C.

Phase transition as detected by spin labeling ESR

For measuring the bulk fluidity of the chloroplast membrane, the C-16 positional isomer of spin-labelled fatty acid (16-SASL) was chosen because this probe selectively monitors the fluidity of the hydrocarbon core which is mostly affected by hydrogenation reactions [31]. At any temperature, in the range between −10°C and 30°C, the ESR spectrum of 16-SASL displayed a single, motionally averaged, isotropic spectral component which was quantitated by calculating the rotational correlation time of the labelled segment. The Arrhenius plots of the rotational correlation time data of the hydrogenated and control samples are shown in Fig. 7. In this representation, the curves of control and hydrogenated chloroplasts show a break at 1°C and 15.1°C, respectively. This shift of discontinuity in the rotational correlation time vs. reciprocal temperature plots is consistent with previously reported changes in fatty acid composition [16]. Though unsaturated fatty acids were subjected only to partial saturation, the removal of double bonds resulted in a marked upward shift of membrane phase transition [30].

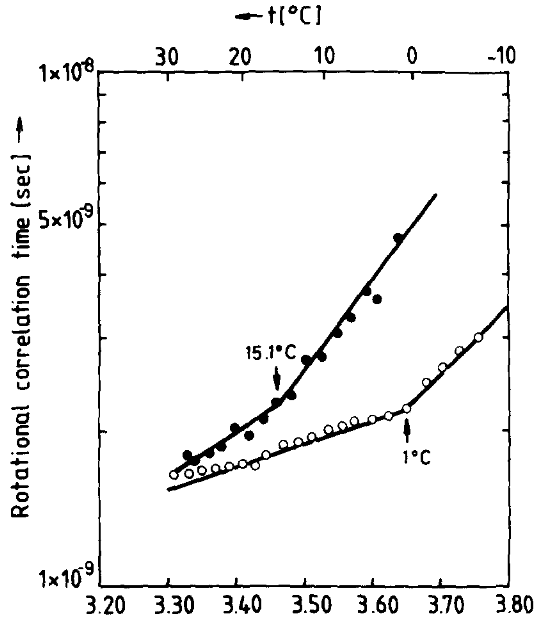

Fig. 7. Arrhenius plots of rotation correlation time of C-16 spin-labelled fatty acids (16-SASL) in chloroplast membranes. The rotation correlation time is used as a measure of bulk membrane viscosity. Untreated chloroplast suspension (○); chloroplasts subjected to 48% saturation of double bonds of membrane lipids (●). Other conditions as in Materials and Methods.

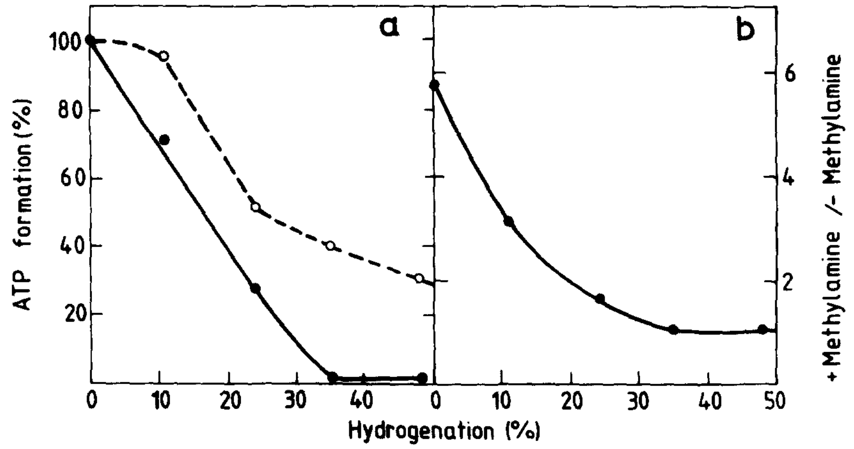

Fig. 8. Effect of hydrogenation on the phosphorylating conditions of isolated chloroplasts. (a) Changes of the activity of photophosphorylation during hydrogenation as measured by the luciferin/luciferase assay system. Noncyclic phosphorylation (●); cyclic phosphorylation measured in the presence of 50 μM PMS (○). (b) Changes in the coupling condition of photosynthetic electron transport during hydrogenation. Whole electron transport was assayed from $H_2O \rightarrow$ methyl viologen. The uncoupled rate of electron transport was measured after addition of 10 mM methylamine. Other conditions as in Materials and Methods.

Photophosphorylation

Both cyclic and non-cyclic phosphorylation showed significant changes upon saturation of the double bonds of fatty acyl residues of membrane lipids (Fig. 8). The capacity of non-cyclic phosphorylation was decreased gradually by progressive hydrogenation, and no ATP formation could be detected when about 35% of double bonds of lipids were saturated (Fig. 8a). It is known that the addition of methylamine to the assay of linear electron transport, measured from $H_2O \rightarrow$ methyl viologen, increases the electron-transport activity by uncoupling phosphorylation from the electron flow [32]. In our experiments, the ratio of the electron transport activity measured from $H_2O \rightarrow$ methyl viologen in the presence and absence of methylamine exhibited a gradual decrease by progressive hydrogenation (Fig. 8b). These results indicate that hydrogenation of double bonds of membrane lipids results in an uncoupling between the non-cyclic phosphorylation and the linear electron-transport flow. Cyclic phosphorylation also showed a partial inhibition when 10% of double bonds of lipids were saturated, but it was only slightly decreased by further hydrogenation (Fig. 8a). This might indicate that the cyclic electron flow is relatively more resistant to hydrogenation than the non-cyclic one and this differential effect of hydrogenation is reflected in H^+ pumping coupled to electron flow. Another explanation could be that the sites of proton translocation, driving cyclic and non-cyclic phosphorylation, are located in different regions of the photosynthetic membrane.

Discussion

The relationship between lipids and photochemical activities in connection with the structural organization of the thylakoid membrane is an intriguing question. The in situ modification of the lipid constituents of the photosynthetic membranes seems to be a promising technique for studying the role of lipids controlling the structure-function relationships of the photosynthetic apparatus. Homogeneous catalytic hydrogenation, introduced by Chapman and Quinn [13], and first applied for thylakoids by Restall et al. [14] makes possible the in situ removal of double bonds of fatty acids in membrane lipids retaining the preexisting architecture of the chloroplast [14,16].

However, some unsatisfactory features of their process (long incubation time, water-insolubility of the catalyst) strictly limit a comprehensive application of this process. The recently developed new homogeneous hydrogenation technique, with $Pd(QS)_2$ catalyst, eliminates the above-mentioned uncertainties [15,16].

In the present study, we demonstrate that, indeed, homogeneous catalytic hydrogenation of lipid constituents of the photosynthetic membranes in situ, induces significant changes in the structural and functional characteristics of these membranes.

We have found that during hydrogenation the basic structure of the chloroplast remains unchanged. Saturation of 10% of fatty acyl double bonds, however, induces a definite decrease in the dimension of both thylakoids and loculi. Thylakoid membrane lipids contain a high proportion of polyunsaturated fatty acid with *cis*-configuration in their hydrocarbon chains [33]. Saturation of *cis*-double bonds by catalytic hydrogenation results in an alignment of the hydrocarbon chain, and consequently increases the ordering state in the deep, hydrophobic region of the membrane [16]. This actual elongation of the fatty acyl chains in a bilayer structure is associated with a thickening of the membrane [34]. Since chloroplast thylakoids consist of two bilayer membranes with an intrathylakoidal space, we expect swelling instead of shrinkage after hydrogenation. Fig. 9 gives a schematic interpretation of this discrepancy. We can assume that, indeed, hydrogenation of double bonds of fatty acyl chains results in a thickening of both single membranes of the thylakoid. However, increased hydrophobicity [6] leads to the loss of the majority of the water located between the two membanes, collapsing the intrathylakoidal space. This seems to be confirmed by the surface potential isotherms of isolated lipid classes which varied in double bond index [35]. Furthermore, the swollen membranes also decrease the dimensions of the interthylakoidal space. As a result, an actual shrinkage of both thylakoids and loculi can be observed. We have found that the shrinkage, induced via hydrogenation, does not alter the orientation pattern of

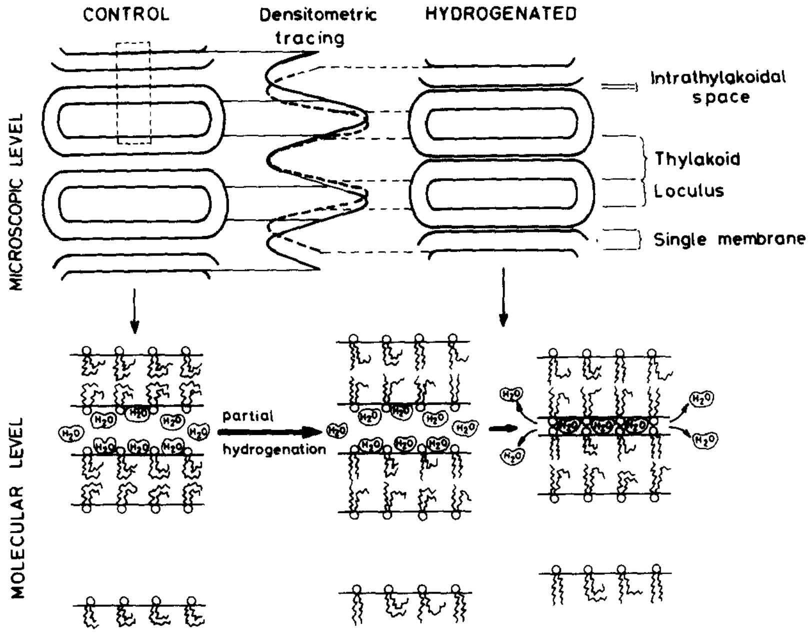

Fig. 9. Proposed mechanism for structural changes in chloroplasts membrane by hydrogenation.

Q_y emission dipoles of chlorophyll *a*. Polarized spectroscopy has shown that Q_y dipoles of long-wavelength forms of chlorophyll *a* lie close to the membrane plane [36], while those of the short-wavelength forms are oriented to a lesser degree [37]. Q_y dipoles of chlorophyll *b* have a tendency to tilt out of the membrane plane [38]. This orientation pattern has been found to be general among plant chloroplasts, although some alteration in the membrane composition might change this orientation pattern [39]. Our result indicates that saturation of about 50% of the fatty acyl double bonds (corresponding to elimination of about 80% of lipid linolenic acid content) does not change the orientation pattern of chlorophyll *a* molecules. Further hydrogenation, specifically, saturation more than 60% of double bonds, increased the fluorescence polarization value for the entire spectrum, especially at 695 nm. It has been proposed by Breton that F_{695} originates directly from the pheophytin (Ph) partner of the P-680-Phe pair when the P^+-680-Phe^- charges recombine [40]. The elevated fluorescence-polarization value of the whole spectrum indicates that extreme hydrogenation might result in a general degradation of the membrane structure which is more pronounced in PS II than in PS I. It must be noted that the inhibitory effect of hydrogenation on photosynthetic electron transport precedes the alteration of the orientation pattern of chlorophylls. Thus, we can conclude that the decreased electron-transport activity obtained by hydrogenation is not a result of the decrease in the efficiency of energy-transfer processes.

We have found that the rate of whole electron transport in hydrogenated chloroplasts is already reduced before either PS II electron transport, measured from $H_2O \rightarrow$ pBQ, or PS I electron flow from DCIP → methyl viologen are inhibited. Early inhibition of PS I electron transport can be observed only when the electron flow is assayed from $DQH_2 \rightarrow$ methyl viologen. It was demonstrated quite recently that the plastoquinone-depleted cytochrome b_6/f complex lost its oxidoreductase activity but could be reconstituted by adding plastoquinone and lipids simultaneously [41]. The addition of phosphatidylcholine and digalactosyldiacylglycerol was especially effective. As a possible explanation, we assume that at the beginning of hydrogenation the polyunsaturated fatty acyl residue of lipids, required for the oxidoreductase activity of cytochrome b_6/f complex, is partially saturated, thus the reduced activity of cytochrome b_6/f complex becomes rate limiting for the whole electron transport. Progressive hydrogenation further reduces the whole electron-transport activity which is followed by a gradually increased inhibition of PS II activity. The fact that the $DQH_2 \rightarrow$ methyl viologen reaction remained constant by further hydrogenation and PS II activity was twice as high as the whole electron transport, suggests that hydrogenation reduces the double bonds of those lipid molecules which ensure the mobility of the electron carrier plastoquinone between the Q_B and cytochrome b_6/f complex [42]. A decreased PS II activity, resulting from hydrogenation, is in agreement with the results of Gounaris et al. [42]. They have found that stimulation of the activity of isolated PS II preparations is dependent on the degree of unsaturation of the lipid used [43]. It is interesting to note that in both PS II preparation and cytochrome b_6/f complex, digalactosyl-diacylglycerol and phosphatidylcholine gave maximal stimulation of electron transport [42,43]. In our experiments, the electron-transport activity showed a linear dependency with the linolenic acid content of the thylakoids. It may be assumed that the linolenic acyl chains of DGDG and PC have important roles in regulating the rate of electron flow.

The temperature dependency of electron transport in uncoupled chloroplasts exhibits discontinuity similar to that described previously [33]. By contrast, the hydrogenated chloroplasts do not show a breakpoint in the temperature range investigated. One possible explanation could be that hydrogenation inhibits uncoupling of phosphorylation and electron flow, since it was reported by Nolan and Smillie that the Arrhenius plots of electron transport were linear as long as chloroplasts were tightly coupled [28,29]. This cannot be the case in our experiments, since the chloroplasts become quickly uncoupled during hydrogenation (cf. Fig. 8a and 8b). On the other hand, we obtained breakpoints int he Arrhenius plots of Hill activity with both coupled and uncoupled, chloroplasts in the control smaples (data not shown). Therefore we believe that the presence or absence of the discontinuity of the Arrhenius plots of Hill activity is more closely related with electron-transport processes than with the coupling phenomenon of photophosphorylation.

Acknowledgements

The authors wish to thank Dr. F. Joó for donating the $Pd(QS)_2$ catalyst, and Mrs. K. Nyerges and Mrs. Zs. Tombácz for skillful technical assistance.

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