Comparative Immunological and Chemical Analysis of Lipids and Carotenoids of the D1-Peptide and of the Light-Harvesting-Complex of Photosystem II of *Nicotiana tabacum*

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The light-harvesting-complex (LHCP) was isolated from photosystem II of *Nicotiana tabacum* var. John William's Broadleaf by means of the detergent acetyl-β-D-glucopyranoside and fractionating centrifugation. The D1-peptide of photosystem II was isolated as a dimer with the molecular mass of 66 kDa from the chlorophyll-deficient tobacco mutant *N. tabacum* Su/su by means of sodium dodecyl sulfate polyacrylamide gel electrophoresis. Both preparations were characterized by means of the Western blot procedure using monospecific antisera to the proteins of photosystem II and monospecific antisera to lipids with which the lipids bound to peptides were determined. In parallel to this, lipids bound to the isolated LHCP-complex and to the isolated D1-peptide were determined by lipido-chemical methods.

The extraction of the isolated core peptide D1 with a mixture of boiling methanol and chloroform and subsequent HPLC-chromatography showed that in the D1-peptide isolated *via* SDS-polyacrylamide gel electrophoresis, monogalactolipids, phosphatidylglycerol and sulfolipid molecules are bound in the molar ratio 1:3:17. By means of the immunological procedure of Western blotting we were able to show that the 66 kDa band of the isolated dimeric D1 reacts positively only with the antisera to monogalactolipid, sulfolipid, β-carotene and violaxanthin. With the antiserum to digalactolipid and that to phosphatidylglycerol a positive reaction is only observed if the preparation used in the Western blot is not the isolated D1-peptide but a "total" photosystem II-preparation.

The lipid extraction of the LHCP-complex and the subsequent analysis by thin-layer chromatography led to the result that the isolated LHCP-complex contained in bound form 3 molecules monogalactolipid, 1 molecule of digalactolipid, 1 molecule of phosphatidylglycerol and 1 molecule of lutein. Less than 1 molecule of sulfolipid, β-carotene, neoxanthin and violaxanthin are found. In the Western blot analysis only the antiserum to monogalactolipid and phosphatidylglycerol and among the carotenoid antisera only the antisera to β-carotene, violaxanthin and to neoxanthin reacted. With the antisera to the digalactolipid, to the sulfolipid and the antisera to the xanthophylls, namely to lutein and neoxanthin, a positive reaction occurred only if the material used in the Western Blot was the "total" photosystem II-preparation.

By gas chromatography of the fatty acids of the isolated peptide fractions it was shown that, compared to the lipids of photosystem II and of the thylakoid membrane, in lipids of the isolated D1-peptide and of the LHCP-complex the saturation degree of fatty acids is strongly increased. Whereas palmitic acid in chloroplast lipids makes up for only 11% of the fatty acids, this saturated fatty acid increases in the lipids of the LHCP to 20% and makes up for 74% of total fatty acids in the lipids of the D1-peptide. Linoleic and linolenic acids are completely absent and oleic acid makes up for 14% of total fatty acids . In contrast to the lipids of the thylakoid membrane, the lipids bound to proteins/peptides are characterized by a strongly saturated character.

Abbreviations:: LHCP, light-harvesting complex; Mes, 2-[N-morpholino]ethane sulfonic acid; PS II, photosystem II; SDS, sodium dodecyl sulfate; Tris, tris[hydroxymethyl]amino-methane; HPLC, high pressure liquid chromatography; MGDG, monogalactosyldiglyceride; DGDG, digalactosyldiglyceride; SQDG, sulfoquinovosyldiglyceride; PAGE, polyacrylamide gel electrophoresis; OEC1, oxygen evolution complex peptide (33 kDa); DTT, dithiothreitol.

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Introduction

In the attempt to determine the function of the thylakoid membrane lipids, the binding of lipids onto peptides of the different functional complexes was determined not only by lipidochemical but also with immunochemical methods. The use of these two methods not always led to identical results. Thus, Murata *et al.* (1990) reported that to the D1/D2/cytochrome b_{559} reaction center complex of spinach monogalactolipid and phosphatidylglycerol are bound and that the oxygen-evolving core-complex of photosystem II besides these two lipids also includes digalactolipid. By immunochemical methods using the Western blot procedure we could show that on the D1-peptide of *Nicotiana tabacum* besides these galactolipids also the two anionic lipids sulfolipid and phosphatidylglycerol are present (Voss *et al.*, 1992a, 1992b; Dädelow *et al.*, 1995). On the D1-peptide of the cyanobacterium *Oscillatoria chalybea* which had been isolated by SDS-polyacrylamide gel electrophoresis, only phosphatidylglycerol and β-carotene were detectable (Kruse *et al.*, 1994; Kruse and Schmid, 1995). These differences are certainly not due to the different plant material but due to the tightness of the lipid-peptide binding and thereby due to the isolation procedure of the peptides and lipids.

On the LHCP-complex of *Nicotiana tabacum* up to now monogalactolipid (Voss *et al.*, 1992a, 1992b), phosphatidylglycerol as well as β-carotene and violaxanthin (Gasser, 1993) were detected by Western blot. To define the function of these lipids bound as prosthetic groups, it is necessary to determine the molar ratio of these lipid-protein complexes. For this purpose the light-harvesting complex of photosystem II of wild type tobacco *N. tabacum* var. JWB and the D1-corepeptide of photosystem II of the *N. tabacum* mutant Su/su were isolated. From these isolated protein fractions the lipids were extracted and analyzed by HPLC- or thin layer chromatography whereas the fatty acid composition was determined by gas chromatography. In parallel to this lipidochemical analysis the lipid and carotenoid binding was determined by Western blotting using monospecific antisera to lipids and carotenoids. These studies showed that detection and determination of certain lipids, such as digalactolipid and the anionic lipids sulfolipid and phosphatidylglycerol strongly depends on the starting material used for Western blot analysis. The present publication describes lipid analyses and the determination of lipid binding to peptides of the photosystem II-complex.

Material and Methods

Isolation of the light-harvesting-complex

For the isolation of the LHCP-complex, PS II-particles were isolated according to Berthold *et al.* (1981) from *N. tabacum* var. JWB. From this preparation following the methods of Ghanotakis and Yocum (1986) and of Specht *et al.* (1987), the LHCP-complex was isolated. For this purpose, the PS II-fractions were taken up in 40 mM Mes-buffer pH 6.0, containing 1 M NaCl, 10 mM $CaCl_2$ and 1 M sucrose and as detergent 100 mM acetyl-β-D-glucopyranoside and were incubated under slight stirring for 15 min at 4 °C. Thereafter, the detergent-containing preparation was supplemented with its two-fold volume of 4 mM Mes-buffer pH 6.0, containing 400 mM NaCl, 5 mM $CaCl_2$ and 1 M sucrose. After 5 min the preparation was centrifuged at 40,000×*g* for 90 min. The LHCP-complexes sedimented, whereas the other free proteins of the PS II-complexes stayed in the supernatant. For further analyses the LHCP-complexes were taken up in 40 mM Mes-buffer pH 6.0, containing 40 mM NaCl and 400 mM sucrose.

Isolation of the D1-peptide

As the D1-peptide occurs in the SDS-polyacrylamide gel electrophoresis as a dimer with the molecular weight of 66 kDa, the protein band was cut out of the SDS-polyacrylamide gel and the peptide eluted from the gel by means of electroelution (Biotrap BT 1000, Schleicher & Schüll).

SDS-polyacrylamide gel electrophoresis and Western blot

The preparations to be analyzed (PS II, LHCP-complex and the 66 kDa peptide) were taken up in incubation buffer (10 mM Tris, pH 8.3, containing 10% glycerol, 2% SDS, 100 mM DTT and 0.01% bromophenol blue) and incubated for 1 h at room temperature. Electrophoresis was carried out with a 12.5% gel. The collection gel contained 3.8% acrylamide. The electrophoresis buffer was

Tris-glycine, containing SDS and urea. The electrophoresis was carried out at 9–10 mA and 4 °C. Western blotting was carried out according to earlier described methods (Voß *et al.*, 1996a; Schmid *et al.*, 1993). The immobilized membrane was nitrocellulose and Immobilon P (Millipore, Bedford). The transfer of peptides was carried out during 30 hs at room temperature or during 60 hs at 4 °C. Since the lipid antisera contain antibodies to methylated bovine serum albumin (MBSA) (Sueoka and Cheng, 1962; Chase, 1967) saturation of the membrane was achieved with 2% fish gelatine solution (Fish gelatine, Sigma).

Antisera

All used antisera were obtained by immunization of rabbits. The lipid and pigment antisera (Radunz 1972, 1976; Radunz and Berzborn, 1970; Radunz and Schmid, 1973, 1975; Radunz and Bader, 1982; Schmid *et al.*, 1993; Lehmann-Kirk *et al.*, 1979a, 1979b) as well as the antisera to the LHCP-complexes of *Nicotiana tabacum* (Dädelow *et al.*, 1995) *Antirrhinum majus* (Schmid *et al.*, 1978) and of the green alga *Chlamydobotris stellata* (Kohnke *et al.*, 1987) have been characterized in earlier publications.

Analysis of lipids

a) For extraction 10 ml suspension of LHCP-complexes was given in 50 ml boiling ethanol and heated for 5 min. Thereafter the particles were further extracted with 25 ml methanol and 25 ml diethylether. The lipids were then partitioned in 10 ml diethylether. From the diethyl ether-soluble lipid mixture a quantitative determination after a thin layer chromatographic analysis on silica gel layers was carried out (Radunz, 1969). Glycolipids were determined *via* a quantitative sugar analysis and phospholipids *via* a quantitative phosphate determination. Carotenoids were separated by thin layer chromatography and photometrically determined. The chlorophylls of the LHCP-complexes were photometrically determined according to Schmid (1971).

b) The lipid extraction of the isolated D1-fraction was first done with 10 ml acetone and subsequently with a mixture of methanol/chloroform (v/v, 1:1). The protein fraction was heated in this solvent for 1 h. After cooling, the proteins were spun down at 15,000×*g*. To verify whether additional lipids or fatty acids were covalently bound to the D1-peptide the fractions extracted with organic solvents were subjected to an alkaline hydrolysis. The proteins were hydrolyzed with 0.5 N methanolic NaOH for 1 h at boiling temperature and subsequently the potentially occurring fatty acids were taken up in petrol ether (40–60 °C b.p.).

c) Fatty acids were determined by gas chromatography *via* the methyl esters (Device from Hewlett Packard, Type 5750). The stationary phase was 10% ethylene glycol- succinate on Chromosorb (Radunz, 1969).

d) The HPLC-analysis was carried out with a device by Hewlett Packard, Böblingen, Germany, on a *reversed phase* column RP 18 (Merck, Darmstadt, Germany). The mobile phase was a mixture of acetonitrile/water/0.5% phosphoric acid, v/v/v, 60/30/40. The samples dissolved in methanol were injected by an *Auto-sampler AS-2000* (Merck u. Hitachi). The flow-rate of the solvent was 0.5 ml/min. The detector used was a diode-detector. Measurements were carried out at 250 nm. The evaluation of the signals was carried out with an integrator. Standard substances which were analyzed under identical conditions were monogalactolipid, digalactolipid, sulfolipid and phosphatidylglycerol.

Results

Isolation and characterization of the LHCPII-complex

The LHCP II-complex was isolated from photosystem II-preparations of wild-type tobacco *N. tabacum* according to the combined procedures of Ghanotakis and Yocum (1986) and Specht *et al.* (1987). The decisive step for the separations of the LHCP II-complex was the incubation of the PS II-preparation with 40 mM Mes-buffer containing 1 M NaCl and 100 mM of the detergent acetyl-β-D-glucopyranoside. During centrifugation at 40,000×*g* the LHCP-complexes are sedimented whereas other peptides of PS II stay in the supernatant. The ratio chlorophyll/protein as well as the lipid/protein ratio of this isolated complex are compared in Table I with PS II-preparations and intact chloroplasts. In isolated LHCP-complexes the chlorophyll/protein ratio was usually found to be

Table I. Ratio of chlorophylls and lipids to protein in the light-harvesting- and photosystem II-complex and in chloroplasts of *Nicotiana tabacum* var. John William's Broadleaf.

Preparation	Chlorophyll: Protein	Chlorophyll *a:b*	Lipid: Protein
LHCP II-complex	1 : 2.2	2.0 : 1	1 : 1.9
PS II-complex	1 : 4	2.3 : 1	1 : 1.9
Chloroplasts of *N.t.* var. JWB	1 : 5	3.2 : 1	1 : 1.2

Light-harvesting complex (LHCP); Photosystem II (PS II); *Nicotiana tabacum* var. John William's Broadleaf (*N.t.* var. JWB). The ratios refer to weight.

1:2.2 (w/w). In isolated LHCP-complexes the chlorophyll/protein ratio as well as the lipid/protein ratio have almost doubled in comparison to chloroplasts.

The purity of the isolated LHCP-complex and the peptide-composition has been analyzed by SDS-polyacrylamide gel electrophoresis (Fig. 1). As the main peptides those with the known molecular weights of 28, 26 and 24 kDa are observed. The peptide with the highest molecular weight appears to be the main component. The LHCP-complex of *N. tabacum* yielded in the Western blot procedure not only cross reactions with antisera to LHCP of other higher plants such as *Antirrhinum majus*, but also with antisera to the LHCP of the green alga *Chlamydobotris stellata* (Fig. 1c–e). In all cases the 3 main proteins of the LHCP of *Nicotiana tabacum* are labelled with the antisera. This means that not only partial identities exist between the LHCP-proteins of higher plants but also between higher plants and green algae. The reactions are shown in Figure 1. Between the LHCP I and LHCP II cross reactions occur as expected.

Chemical analysis of the lipids of the LHCP-complex

A quantitative lipid analysis showed that the lipids extractable with ethanol, methanol and diethyl ether make up for 34.5% of the LHCP-preparation. The qualitative and quantitative composition of the lipids was determined by thin layer chromatography. Glycolipids were analyzed *via* the determination of the sugar portion and phospholipids *via* determination of the phosphate portion. In Table II the composition of these lipids is compared with that of the PS II-fraction and that of intact stroma-freed chloroplasts from *Nicotiana tabacum* var. JWB. As expected chlorophylls make up for 68% of total lipids. Whereas chlorophyll *a* increases in comparison to chloroplasts 2.3-fold in the LHCP-lipids, chlorophyll *b* increases 3.4-fold. Concerning carotenoids the picture looks dif-

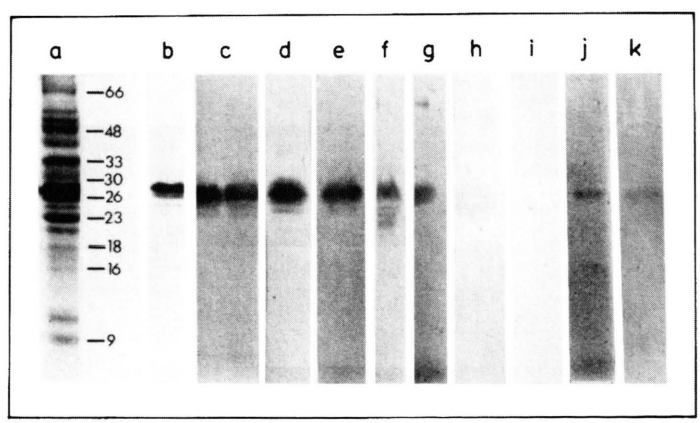

Fig. 1. Characterization of the LHCP-complex of *Nicotiana tabacum* var. JWB with homologous and non-homologous antisera, as well as with antisera to lipids and carotenoids in the Western-Blot-procedure.
a) Photosystem II preparation of *N. tabacum* var. JWB after SDS-polyacrylamide gel electrophoresis (PAGE); b) isolated LHCP-complex after SDS-PAGE; c–k) Nitrocellulose membranes with the peptides of the LHCP-complex after reactions with c) a homologous antiserum; d) an antiserum to the LHCP-complex of *Antirrhinum majus*; e) an antiserum to the LHCP-complex of the green alga *Chlamydobotris stellata*; f) an antiserum to monogalactolipid; g) an antiserum to phosphatidylglycerol of chloroplasts; h) an antiserum to synthetic phosphatidylglycerol, containing exclusively palmitic acid; i) a control serum; j) an antiserum to β-carotene; k) an antiserum to violaxanthin.

Table II. Lipid composition of the light-harvesting- and photosystem II-complex as well as that of chloroplasts of *Nicotiana tabacum* var. JWB.

Lipids	LHCP II % of lipids	PS II % of lipids	Chloroplasts % of lipids
MGDG	15.3	24.0	32.0
DGDG	7.0	9.9	25.9
SQDG	0.9	2.9	7.4
PG	3.2	1.1	1.9
PC	–	2.3	2.1
PI	–	0.11	0.46
Chlorophyll *a*	45.6	36.8	20.3
Chlorophyll *b*	21.6	15.9	6.3
Lutein	4.5	4.0	1.7
Neoxanthin	1.2	1.6	0.6
Violaxanthin	0.4	0.6	0.4
β-Carotene	0.3	0.8	0.9
Ratio			
MGDG : DGDG	2.2	2.4	1.2
MGDG + DGDG : SQDG	24.2	11.7	7.8
MGDG : chlorophyll	0.23	0.46	1.20
Carotenoids : chlorophylls	0.10	0.13	0.14

MGDG, monogalactosyldiglyceride; DGDG, digalactosyldiglyceride; SQDG, sulfoquinovosyldiglyceride; PG, phosphatidylglycerol; PC, phosphatidylcholine; PI, phosphatidylinositol.

ferent. A comparison makes only sense if the LHCP-carotenoids are compared with the PS II-carotenoid pattern. Only lutein increases in the LHCP-lipids whereas β-carotene as well as the two xanthophylls violaxanthin and neoxanthin decrease. Possibly the carotenoids which are stronger represented in the PS II-preparation than in the LHCP are bound to the LHCP and to the core peptides and the chlorophyll binding peptides as well.

As shown by HPLC-analysis, the glycolipids MGDG, DGDG and SQDG decrease in comparison to the PS II-complex and to chloroplasts. However, as the strong increase of the chlorophylls in the PS II- and light-harvesting lipids strongly "represses" the colourless lipids, a comparison seems only reasonable if in these fractions the relative ratio of the pigment-free lipids is compared (Table II). Only then the different behaviour of the galactolipids is seen. Whereas the monogalactolipid of photosystem II and of the light-harvesting complex (LHCP) increases in comparison to chloroplasts, the digalactolipid decreases. The sulfolipid appears decreased in the LHCP by almost ⅔ and phosphatidylcholine and phosphatidylinositol do not occur anymore. The strongest shift is seen with phosphatidylglycerol. This lipid is increased in the LHCP-complexes almost 5-fold. Hence, phosphatidylglycerol is the only phospholipid occurring in the LHCP. Table III shows the molar ratio of lipids and pigments per 28 kDa peptide (which is the peptide of the complex with the highest molecular mass). Whereas 11 chlorophyll molecules are counted per 28 kDa-peptide, only 4 galactolipid molecules are present in this peptide. Concerning phosphatidylglycerol and lutein a molar ratio with the 28 kDa peptide of 1:1 is observed. Concerning β-carotene, violaxanthin and neoxanthin as well as the sulfolipid only every 10th peptide molecule binds 1 β-carotene or violaxanthin or every 3rd peptide binds 1 neoxanthin molecule. If the number of lipid and pigment molecules is compared per peptide molecule and if molar masses of lipids and the proteins are compared it becomes evident that lipids make up for ½ of the protein mass. From this one can conclude, that the here quantified and extracted lipids are molecules which are only loosely adsorbed onto protein or protein- complexes *via* intermolecular binding forces. These lipids are extractable by ethanol, methanol and ether. One can also say that in the SDS-gel electrophoresis the major amount of lipids is detached. Otherwise the properties of the protein molecules would appear drastically changed by lipid masses half as high as those of the protein. Ryrie *et al.* (1980) determined the molar ratio of lipids to the 26 kDa-peptide in the LHCP of spinach (Table III) and obtained the same result. A discrepancy exists for chlorophyll *a*.

Table III. Number of lipid and pigment molecules per 28 kDa peptide of LHCP II of *N. tabacum* (JWB) and corresponding values per 26 kDa peptide of LHCP II of *Spinacia oleracea* (Ryrie *et al.*, 1980).

Lipid	LHCP II (*N.t.* JWB)	LHCP II (*S. oleracea*)
Chlorophyll a	7.5	3.7
Chlorophyll *b*	3.5	3.1
MGDG	2.9	2.7
DGDG	1.2	1.4
SQDG	0.15	0.8
PG	0.63	0.3
Lutein	1.17	1.0
Neoxanthin	0.30	0.57
Violaxanthin	0.10	0.26
β-Carotene	0.07	0.05

MGDG, monogalactosyldiglyceride; DGDG, digalactosyldiglyceride; SQDG, sulfoquinovosyldiglyceride; PG, phosphatidylglycerol.

The LHCP of *Nicotiana tabacum* contains a 2-fold higher number of molecules than spinach. Burke *et al.* (1979) determined the number of chlorophyll molecules per LHCP-peptide in barley and found 13 molecules, Butler and Kühlbrandt (1988) found 15.5 molecules.

The gas chromatographic analysis of the fatty acids led to the result, that the fatty acids are characterized by a higher portion of saturated fatty acids (Table IV). Whereas the saturated fatty acids make up for 12% in chloroplasts and 11% in photosystem II, they increase in the LHCP-lipids to 21%. Palmitic acid increases 4-fold in comparison to photosystem II-lipids. Whereas linolenic acid decreases by 13%, the three-fold unsaturated hexadecatrienoic acid with 16 carbon atoms, also occurring as ester component in monogalactolipids, stays practically constant. Hence, the ratio of fatty acids with 16 carbon atoms to those of 18 carbon atoms is shifted in the LHCP-lipids in favour of fatty acids with 16 carbon atoms. Basically this means that the lipids of the LHCP of photosystem II have a substantially lower fluidity than lipids of the photosystem II-fraction. A further characteristic of LHCP-lipids is the fact that hexadecenoic acid with trans-configuration increases two-fold. However, phosphatidylglycerol which is bound to this acid increases 5-fold. This means that in the LHCP-lipids also phosphatidylglycerol molecules occur which have less trans-hexadecenoic acid than usual.

Table IV. Fatty acid composition of the lipids of the core peptide D1 of LHCP II- and the PS II-complex as well as that of chloroplasts of *N. tabacum* var. JWB calculated as per cent of total fatty acids.

Fatty acids	D1-peptide	LHCP II	PS II	Chloroplasts
$C_{12:0}$	–	–	2.5	0.6
$C_{14:0}$	–	0.9	3.0	0.3
$C_{16:0}$	74.0	19.7	5.2	11.3
$C_{16:1\ trans}$	4.5	6.9	3.2	4.3
$C_{16:2}$	8.0	–	1.5	1.3
$C_{16:3}$	–	11.5	13.5	9.7
$C_{18:1}$	13.5	2.0	1.4	3.0
$C_{18:2}$	–	2.4	4.1	6.8
$C_{18:3}$	–	56.6	65.6	62.7
C_{16}	86.5	38.1	23.4	26.6
C_{18}	13.5	61.0	71.1	72.5
Saturated fatty acids	74.0	20.6	10.7	12.2

Immunological detection of lipid-binding to the LHCP-complex

It was shown by Western-blotting, using antisera to lipids that the isolated LHCP-complex reacts positively with the antiserum to monogalactolipid and also with that to phosphatidylglycerol. In the reaction with monogalactolipid it becomes evident that here a labelling of 3 respectively 4 LHCP-bands occurs. This result demonstrates the purity of the isolated LHCP-complex and shows that all peptides of the LHCP contain bound monogalactolipid. Whereas this antiserum reacts in a photosystem II-preparation besides with the LHCP-peptides also with the OEC1, 2 and 3 peptide as well as with D1 and D2, with the *isolated* LHCP-preparation only the peptides of this complex are seen. With the antiserum to phosphatidylglycerol only the two main bands of the LHCP-complex with the molar masses 28 and 26 kDa are labelled (Fig. 1g, h). An antiserum to phosphatidylglycerol which is directed towards a synthesized phosphatidylglycerol containing only palmitic acid in contrast to the antiserum to the natural plant lipid containing trans-hexadecenoic acid, showed only a weak reaction, despite the fact that fatty acids do not represent antigenic determinants.

The antiserum to the digalactolipid and that to the sulfolipid gave with the light-harvesting complex only a reaction if a photosystem II-preparation was used. With the isolated LHCP-complex no positive reactions were observed anymore and this despite the fact that the quantitative chemical analysis showed, that the molar ratio of digalactolipid to protein of the LHCP-complex was 1:1. This observation demonstrates that during the Western-Blot procedure under the influence of SDS, digalactolipid is split off. With the carotenoid antisera we were able to show that only the antiserum to β-carotene and to violaxanthin reacts positively with the LHCP-proteins. The antiserum to lutein and that to neoxanthin did not react. For the two carotenoids either the same explanation as for the digalactolipid is valid or these two xanthophylls are located in a site which is inaccessible to antibodies.

Detection of lipids on the D1-peptide

As the D1-peptide occurs in the SDS-polyacrylamide gel electrophoresis of photosystem II prep-

arations of the *N. tabacum* mutant Su/su preponderantly as a dimer with the molecular weight of 66 kDa, the isolation of this peptide was performed *via* this 66 kDa-aggregate. Moreover, this isolation has the advantage that no impurities by LHCP-peptides, by the D2-peptide or by OEC1 can occur. The peptide was isolated *via* electroelution from the gel. As demonstrated by Western-blotting, the D1-peptide was prepared by this procedure in pure form (Fig. 2c). Thus, it reacts exclusively with the D1-antiserum and gave no cross reactions with the antiserum to the D2-, LHCP- or OEC1-peptides of photosystem II. This was taken as the proof that pure D1-peptide was isolated. As shown in Fig. 2d+c the isolated D1 reacts with an antiserum to D1 as well as with an antiserum to photosystem II in the region of the D1-monomer (with a molecular weight of 32 kDa) but also with the dimer (with a molecular weight of 66 kDa). If the experiment is started in the Western-blot with a photosystem II-preparation and if this preparation is brought to reaction with the anti-D1-serum a reaction with the monomeric D1 and with a peptide band in the region of approx. 18 kDa is observed. Whereas the 66 kDa peptide is an aggregate *i.e.* a dimer of D1, the 18 kDa peptide appears to be a fragment of D1 (Nanba and Satoh, 1979; Greenberg *et al.*, 1987, Wälzlein and Pistorius, 1992).

It was shown by Western-blotting and using monospecific antisera to lipids, that this D1-peptide isolated *via* SDS-gel electrophoresis, contains as galactolipids only monogalactosyldiglyceride and sulfoquinovosyldiglyceride in bound form (Fig. 2). The reactions with the antiserum to the digalactolipid and that to phosphatidylglycerol and isolated D1 came out negatively. This was true despite the fact that the 66 kDa-peptide as well as the chlorophyll binding peptides and the peptides of the LHCP-complex were labelled with 3 different antisera to digalactolipids provided that in the Western-blot procedure the untreated total photosystem II-fraction was analyzed and transferred to nitrocellulose. The reaction of the 66 kDa peptide is due to the fact that during the purification procedure of the peptide the digalactolipid is split off whereas the monogalactolipid and the sulfolipid are apparently tighter bound.

For further identification of these bound lipids the isolated D1-aggregate was extracted for 90 min at boiling temperature with a mixture of methanol/chloroform (1:2, v/v) with the extract being analyzed by HPLC on a RP-18-reversed phase column. By this procedure the immunologi-

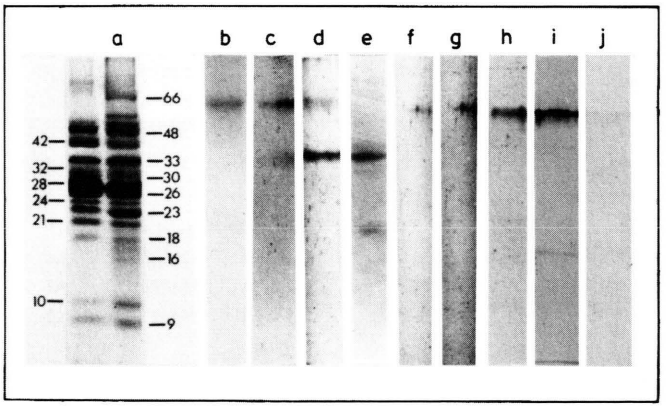

Fig. 2. Reactions of the D1-core peptide of photosystem II from the *Nicotiana tabacum* mutant Su/su in the Western-blot with antisera to proteins and lipids. a) peptide composition of two photosystem II preparations of the *Nicotiana tabacum* mutant Su/su after SDS-PAGE with different amounts of the aggregated D1-peptide in the region of the 66 kDa-band; b) isolated D1-peptide after SDS-PAGE; c-j) Nitrocellulose membranes with the D1-peptide after reactions with c) isolated D1-peptide with the homologous antiserum; d) isolated D1-peptide with PS II-antiserum; e) PS II-preparation with D1-antiserum; f) isolated D1-peptide with an antiserum to monogalactolipid; g) isolated D1-peptide with an antiserum to sulfolipid, h) PS II-preparation with an antiserum to digalactolipid; i) PS II-preparation with an antiserum to phosphatidylglycerol, j) PS II-preparation with an antiserum to synthesized phosphatidylglycerol.

cally detected lipids monogalactolipid and sulfolipid were identified as well as phosphatidylglycerol. The occurrence of digalactolipid was not confirmed by HPLC-chromatography. A quantitative analysis of the HPLC-chromatogram showed, that the detected lipids monogalactolipid, phosphatidylglycerol and sulfolipid are contained in a ratio 1:3:17. This result shows that in the course of isolation of the core peptide the digalactolipids are split off, and that considerable amounts of sulfolipid are adsorbed unspecifically which, however, can only be removed by boiling methanol/chloroform. A gas chromatographic analysis of the fatty acids of this lipid mixture bound onto the D1-peptide, led to the result that palmitic acid as the major component makes up for 74% of total fatty acids. Oleic acid makes up for 14% and a dienoic acid with 16 carbon atoms for 18% of total fatty acids. In the course of this gas chromatographic analysis a C_{16} monoenoic acid with transconfiguration typical for phosphatidylglycerol was detected. This acid makes up for only 4.5%. Surprisingly the highly unsaturated fatty acids linolenic and linoleic acid, in general typical for the detected lipids monogalactolipid, sulfolipid and phospholipid, were not identified. This means that the lipids bound onto the D1-peptide exhibit a characteristic difference in their fatty acid composition in comparison to the anionic lipids and the monogalactolipid of the thylakoid membrane. In order to verify whether further lipids and fatty acids are chemically bound onto the D1-peptide, the isolated D1-peptide extracted with the methanol/chloroform mixture was subjected to an alcaline hydrolysis with the potentially occurring hydrolysis products being taken up with a mixture of petrolether/diethyl ether (1:1, v/v). The gas chromatographic analysis showed that by alkaline hydrolysis no further fatty acids were split off.

Discussion

The present publication compares results on the binding of lipids and pigments on the D1-peptide and the LHCP-complex of photosystem II obtained by immunology with those obtained by analytical chemical methods. Western-blot analyses have shown that for the LHCP-complex as well as for the D1-peptide reactions with lipid and carotenoid antisera depend on the type of preparation used for the SDS-gel electrophoresis. A D1-peptide, isolated *via* SDS-gel electrophoresis, reacts in the Western-Blot procedure only with the antiserum to monogalactolipids or that to sulfolipid. An antiserum to digalactolipid or phosphatidylglycerol reacts with the D1-peptide in a positive way only if a "total PS II-"preparation is used.

As HPLC-analysis of the lipids extracted with boiling methanol/chloroform from the D1-peptide has shown that the D1-peptide isolated *via* SDS gel electrophoresis contains as bound lipids only monogalactosyldiglyceride, phosphatidylglycerol and sulfolipid in a ratio of 1:3:17, it is concluded that a difference has to be made between lipids like monogalactolipid and phosphatidylglycerol which are very tightly bound and lipids like digalactolipid which are only loosely adsorbed. These two types must be further distinguished from lipids like the anionic sulfolipid, which as shown by HPLC-analysis, due to their negative charge, get tightly adsorbed in the course of the isolation procedure, remain adsorbed and are absolutely not split off during the SDS-treatment. With an artificial emulsion of ovalbumin and a lipid mixture of monogalactolipid, digalactolipid and sulfolipid we were indeed able to show that these lipids get very tightly bound to the respective albumin, and are not split off in the course of the SDS-gel electrophoresis nor in the subsequent Western-blotting (unpublished).

The molecules of phosphatidylglycerol must have undergone, during the D1-isolation and the therewith connected partial denaturation and aggregation, a molecular structural change leading to the result that antigenic determinants (in the glycerol-phosphate region) are not anymore accessible to antibodies.

In the light-harvesting complex obtained by detergent treatment and subsequent fractionating centrifugation, the three glycolipids MGDG, DGDG and SQDG, phosphatidylglycerol and the four carotenoids β-carotene, lutein, violaxanthin and neoxanthin were detected by means of thin layer chromatography. A comparison of the molar ratio between lipids and the main peptide of the LHCP-complex with the molecular mass of 28 kDa shows that approx. 4 glycolipids, 1 phosphatidylglycerol and 2 carotenoids as well as 11 chlorophyll molecules are bound to 1 peptide. Immunological techniques *e.g.* the Western blot

gave positive reactions only with the antiserum to the monogalactolipid and that to phosphatidylglycerol and the antisera to the two carotenoids β-carotene and violaxanthin. The isolated LHCP-complex does not react anymore with the antiserum to the digalactolipid nor with that to sulfolipid or the xanthophyll antisera lutein and neoxanthin. The Western blot with these lipid and xanthophyll antisera was only positive if the analyzed preparation was a "total photosystem II-complex"-preparation.

Moreover, due to the differing results obtained by immunological analysis on the one side and by chemical analysis on the other side it must be assumed for the LHCP-complex, that the lipids are located in cleft-like deepenings, as it was shown for the localization of fatty acids in fatty acid-transport proteins, which means a steric hindrance for the antibody reactions. The other interpretation would be that the lipids digalactolipid, sulfolipid as well as the xanthophylls lutein and neoxanthin have suffered in the course of the isolation procedure, – due to peptide folding, *i.e.* partial denaturation –, a molecular rearrangement in the sense that the antigenic determinants of these components are not accessible anymore to antibodies. In test reactions with a glycosyl-phosphatidylinositol anchor protein neither in the Dot-blot nor in the Western-blot procedure a positive reaction with an antiserum to phosphatidylinositol was obtained. Even after protein decomposition with pepsin or trypsin the reactions remained negative (Berwis *et al.*, 1995).

Due to the fact that lipids like the digalactolipid are only loosely bound or adsorbed to proteins and that lipids like the sulfolipid, due to their negative charge are secondarily adsorbed onto certain proteins during the isolation procedure, one should start from the point of view that during isolation procedures protein preparations may be obtained which differ in their composition with respect to these lipids.

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