Immunological Evidence for the Binding of β-Carotene and Xanthophylls onto Peptides of Photosystem I from *Nicotiana tabacum*

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Photosystem I preparations were obtained from wild type tobacco *Nicotiana tabacum* var. John William's Broadleaf (JWB) and from the two chlorophyll-deficient mutants *N. tabacum* Su/su and *N. tabacum* Su/su var. Aurea. The preparations were characterized with respect to the chlorophyll *a/b* ratio, their photosynthetic activity and their absorption spectroscopic properties. Peptides from these preparations were analyzed by SDS polyacrylamide gel electrophoresis and transferred for the detection of bound carotenoids according to the Western blot procedure to nitrocellulose or Immobilon membranes. The PS I preparation from the wild type JWB consisted of the core and the LHCP complex. The core complex contains the two core peptides with the same apparent MW of 66 kDa and several peptides with the lesser molecular masses of 22, 20, 19, 17, 16, 10 and 9 kDa. The light-harvesting protein complex consists of 4 subunits with the molecular masses 28, 26, 25 and 24 kDa. The PS I preparations of the yellow-green mutant Su/su and of the Aurea mutant Su/su var. Aurea contain as impurity traces of the D_1 and D_2 core peptides of photosystem II and also traces of the chlorophyll-binding photosystem II peptides with the molecular masses 42 and 47 kDa.

The peptides of the photosystem I preparation were characterized by specific photosystem I antisera: An antiserum to the photosystem I complex reacts in the Western blot only with the homologous peptides of photosystem I. In comparative analyses with photosystem II preparations this antiserum (directed to photosystem I) reacts, as expected, only with the peptides of the light-harvesting complex. An antiserum to the CP1 core peptides reacts only with the 66 kDa peptides of photosystem I and gives no cross reaction with heterodimer forms of the D_1/D_2 core peptides of photosystem II.

In the Western blot procedure by means of polyclonal monospecific antisera to carotenoids it was demonstrated that β-carotene is bound in high concentration onto the core peptides CP1 and to a lesser extent onto the two larger subunits of the LHCP complex, exhibiting the molecular masses of 28 and 26 kDa. Neoxanthin is bound onto the same peptides. In contrast to this, lutein was only identified on the core peptides CP1 and violaxanthin only on the larger subunits of the LHCP complex. As the carotenoids are labelled with antibodies, even after SDS treatment in the electrophoresis, it is assumed, that the carotenoids are covalently bound *via* the ionon ring to the respective peptide.

Introduction

The main function of carotenoids occurring in the thylakoid membrane, consists in the absorption of light energy and transfer of the absorbed energy to the reaction centers (Boucher *et al.,* 1977; Searle *et al.,* 1978; Siefermann-Harms, 1980, 1985; Cogdell, 1985, 1988) and in the protection of chlorophylls and proteins of the reaction centers of PS I and PS II against photooxidation (Krinsky, 1979; Schrott, 1985; Siefermann-Harms, 1987; Damm *et al.,* 1987; Cogdell, 1985, 1988). Accordingly, the carotenoids are differently distributed between the functional complexes. Whereas β-carotene is, as shown by chemical studies, mainly located in the chlorophyll-protein complexes of PS I and PS II, the xanthophylls lutein, violaxanthin and neoxanthin are more concentrated in the light-harvesting complexes (Siefermann-Harms, 1980, 1985; Damm *et al.,* 1987; Braumann *et al.,* 1982; Damm *et al.,* 1990). As to SDS treatment and SDS gel electrophoresis the major portion of

Abbreviations: PS I, photosystem I; LHCP, light harvesting chlorophyll protein complex; DCPIP, dichlorophenolindophenol; transfer buffer, 10 mM Tris, pH 8.8, 2 mM EDTA, 50 mM NaCl; STMN, 0.05 M Tris, pH 7.8, 0.04 M sucrose, 0.01 M NaCl, 5 mM $MgCl_2$; McIlvaine buffer, 50 mM $C_6H_8O_7 \cdot H_2O$ (citric acid monohydrate), 50 mM $Na_2HPO_4 \cdot H_2O$, pH 3.

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these hydrophobic pigments occurs as free pigment (Siefermann-Harms, 1980, 1985). This means that these carotenoids are not bound to the chlorophyll-protein complexes but are only adsorbed by intermolecular interaction. Only 20% of the carotenoids appear to be bound to proteins of the photosystem I and II complexes and to the light-harvesting complexes (Siefermann-Harms, 1980).

The functional characterization of carotenoids in the photosynthetic electron transport chain by means of specific antisera had already been attempted 15 years ago (Radunz and Schmid, 1973, 1975, 1979; Lehmann-Kirk *et al.*, 1979a, b; Radunz and Bader, 1982). These antisera inhibited photosynthetic electron transport reactions in the region of photosystem I and photosystem II always on the respective donor side (Fig. 1). The extent of the inhibitory effect depended on the osmotic condition of the thylakoid membrane, on the amount of antibodies used, as well as on the temperature and the pH of the reaction medium (Radunz and Schmid, 1973, 1975, 1979, 1988; Lehmann-Kirk *et al.*, 1979a, b; Radunz and Bader, 1982). These observations obtained by serological means, suggested, that functionally active carotenoids are bound onto peptides of the electron transport chain in the region of both photosystem I and photosystem II.

Correspondingly, Nanba and Satoh have shown in 1986 that core complexes of photosystem II from spinach which were composed of the D_1/D_2 core peptides and cytochrome b_{559}, contained besides chlorophyll also one or two molecules of β-carotene (Nanba and Satoh, 1987). This observation was confirmed by other authors with core complexes from other plants (Barber *et al.*, 1987; Kobayashi *et al.*, 1989; Shuvalov *et al.*, 1989; Montoya *et al.*, 1991; Braun *et al.*, 1990). By means of studies with the Western blot procedure we were able to show that glycolipids, whose antisera also affect electron transport reactions in a similar way (Radunz *et al.*, 1984a, b), are bound to the D_1/D_2 core peptides of photosystem II (Voß *et al.*, 1992). In the present study we report on the binding of β-carotene and that of xanthophylls, occurring in higher plants, to the core peptides of photosystem I and peptides of the light-harvesting complex of photosystem I of *Nicotiana tabacum* var. JWB and of the yellow-green and the yellow tobacco mutant Su/su and Su/su var. Aurea. This plant material was chosen because chloroplasts of these tobacco species exhibit differences in the morphology of their lamellar system and in their photosynthetic activity (Schmid *et al.*, 1966; Schmid, 1967; Okabe *et al.*, 1977; Radunz and Schmid, 1989). Whereas the lamellar system of chloroplasts from the wild type is composed of grana and intergrana regions, the lamellar system of chloroplasts of aurea mutants has mainly stroma lamellae with occasional doublings whereas in Su/su chloroplasts the grana regions are strongly reduced.

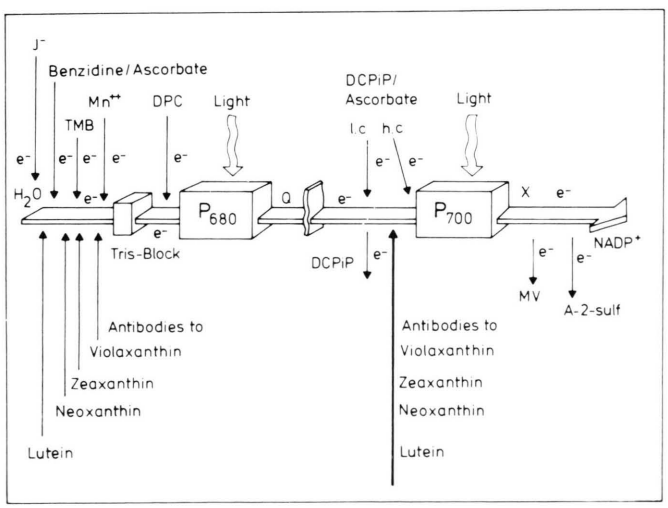

Fig. 1. Electron transport scheme with indication of the inhibition sites of electron transport on the donor sides of photosystem I and photosystem II by monospecific polyclonal antisera to carotenoids. (From Schmid, Radunz and Gröschel-Stewart. Immunologie und ihre Anwendung in der Biologie. Georg Thieme Verlag, Stuttgart, New York 1993.)

Materials and Methods

Plant material and photosystem I preparations

Photosystem I was isolated from *N. tabacum* var. JWB and the two chlorophyll-deficient mutants Su/su and Su/su var. Aurea (Schmid *et al.*, 1966; Schmid, 1967; Okabe *et al.*, 1977). The plants were grown in an air-conditioned growth chamber with a light/dark cycle of 16/8 h at a day temperature of 27 °C and constant relative humidity at 60%. PS I-200 preparations from the three tobacco varieties were prepared according to Wynn and Malkin (1988). Tobacco leaves were homogenized at 4 °C in cold STMN (0.4 M sucrose, 0.05 M Tris-HCl, pH 7.8, 0.01 M NaCl and 5 mM $MgCl_2$). The homogenate was filtered through four layers of nylon mesh and the filtrate was centrifuged at 600×*g* for 5 min. The supernatant was then centrifuged for 10 min at 2000×*g* to pellet the chloroplasts. The pellet was resuspended in 100 mM NaCl and incubated for 10 min at 4 °C followed by centrifugation at 4000×*g* for 15 min. The chloroplast membranes were then resuspended in STMN which contained 2 M NaBr and were incubated for 30 min at 4 °C followed by dilution with twice the volume of STMN and centrifuged at 4000×*g* for 20 min. The pelleted membranes were resuspended twice in STMN and once in 0.05 M Tris-HCl buffer, pH 7.8 and centrifuged at 4000×*g* for 10 min. By addition of Tris-HCl buffer, pH 7.8, the chlorophyll concentration was adjusted to 1 mg/ml, and Triton X-100 from a 20% stock solution was added in order to give a final concentration of 1%. This assay was incubated for 1 h at 4 °C followed by centrifugation at 48,000×*g* for 30 min to remove unsolubilized material.

The supernatant containing solubilized PS I and LHCP complexes was loaded onto a 0.4–1 M sucrose gradient which contained 0.02% Triton X-100 with a 2 M sucrose cushion and was centrifuged at 100,000×*g* for 18 h at 4 °C. The dark-green band at the top of the 2 M cushion contained the native PS I-200 particles (Fig. 2).

Chlorophyll was determined according to Schmid (1971) in methanol/water 90/10 (v/v) and protein determinations were done according to Bradford (1976).

SDS polyacrylamide gel electrophoresis

PS I polypeptides were modified in the SDS polyacrylamide gel electrophoresis according to the methods of Weber and Osborn (1969) and Laemmli (1970). A 1.5 mm gel with a 10–20% gradient separation gel and a 3% collection gel was used. Prior to electrophoresis, samples were solubilized with 200 mM DTT, 2% SDS and 20 mM Tris-HCl buffer, pH 8.4 at 50 °C for 20 min. Electrophoresis was carried out at a constant current of 25 mA for 6 h at 4 °C. Following electrophoresis, the gel was stained with Coomassie Brilliant Blue.

Western blotting

Western blotting was performed as described by Renart *et al.* (1979). The proteins were transferred by pressure from SDS gels to nitrocellulose membranes during 20 h at room temperature. The nitrocellulose was blocked with 2.5% fish gelatine. The concentration of the specific carotenoid antisera is given in the respective figure legends. The second antibody, peroxidase-conjugated pig immunoglobulins against rabbit immunoglobulins (anti-rabbit IgG, DAKO), was diluted 100-fold. Specifically bound antibodies were stained by the reaction of peroxidase with H_2O_2 and 4-chloro-1-naphthol (Sigma).

Conditioning of the PS I preparations and of the proteins transferred to the Immobilon-P membranes

For demonstration of carotenoid-binding onto peptides, the above described PS I preparations were subjected to SDS polyacrylamide gel electrophoresis. In order to determine the binding mode of the carotenoids, the PS I preparations were treated in parallel before the SDS gel electrophoretic analysis with acetone, in order to extract the major part of the soluble carotenoids. For this purpose, 1 ml of a PS I suspension, corresponding to a concentration of 1 mg/ml chlorophyll, was supplemented with 10 ml acetone and incubated for 1 h at 4 °C. The PS I particles were subsequently centrifuged at 4000×*g* and resuspended in 0.05 M Tris buffer, pH 7.8.

Treatment of the Immobilon-P membrane with McIlvaine buffer was carried out as follows: After

the peptide transfer the Immobilon-P membranes were washed with transfer buffer and subsequently incubated with McIlvaine buffer at room temperature. The buffer consisted of 50 mM citric acid and 50 mM $Na_2HPO_4 \cdot 2H_2O$ having pH 3. Thereafter, the membranes were washed with transfer buffer and incubated with the respective carotenoid antisera.

Antisera

Monospecific polyclonal antisera to β-carotene and to the xanthophylls lutein, violaxanthin and neoxanthin were obtained by immunization of rabbits (Radunz and Schmid, 1973, 1975, 1979; Lehmann-Kirk *et al.*, 1979a, b; Radunz and Bader, 1982). β-Carotene and lutein were purchased as synthetic compounds from Hoffmann-La Roche, Basel. Violaxanthin and neoxanthin were isolated from *Urtica dioica* and purified over powdered sugar columns and thin layer chromatography on $Mg(OH)_2 \cdot CaCO_3$ silica gel layers. The obtained antisera exhibited different activities. The high specificity of the antisera was derived from the specifically different inhibitory action of photosynthetic electron transport reactions. As shown in Fig. 1 we were able to show that the carotenoid antisera inhibited photosynthetic electron transport on distinctly different sites on the donor side of PS II. For the preparation of the antisera to the CP1 proteins the two core peptides were isolated by SDS polyacrylamide gel electrophoresis from the PS I preparation of the wild type *N. tabacum* and by electroelution from the gel. The antiserum was monospecific and reacted only with the CP1 core peptides from PS I preparations. Correspondingly, a PS I antiserum reacted only with peptides of the PS I preparation and cross-reacted only with peptides of the light-harvesting complex of photosystem II preparations.

Results

Characterization of PS I preparations

PS I preparations were prepared according to Wynn and Malkin (1988) from wild type tobacco and from the two chlorophyll-deficient mutants Su/su and Su/su var. Aurea. These preparations were further purified by 18 h centrifugation at 100,000×*g* over a linear 0.4–1 M sucrose density gradient. The lower green band of the gradient contained the native PS I preparation (Fig. 2).

The chlorophyll *a/b* ratio of the PS I preparation from the wild type was on the average 4.0. Due to the strong reduction of the LHCP complex in the mutants, the chlorophyll *a/b* ratio decreases in the mutant to 3.5 and in the mutant Su/su var. Aurea to 3.0 (Table I).

The photosynthetic activity of the obtained PS I preparation is depicted in Table I. Electron transport was measured as methylviologen reduction with the electron donor couple DCPIP/ascorbate. The activity of the preparation from wild type tobacco corresponds to the order of magnitude of that from spinach. In the course of the purification

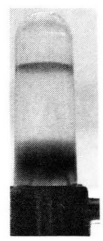

Fig. 2. Linear 0.4–1 M sucrose gradient (underlaid with a 2 M sucrose cushion).

Table I. Photosynthetic activity and chlorophyll *a/b* ratio of photosystem I preparations from *Nicotiana tabacum* siblings and *Spinacia oleracea*. Photosynthetic activity was measured as the reduction of methylviologen with the electron donor couple DCPIP/ascorbate *via* a Mehler reaction with a Clark type electrode from Rank Brothers (England). The methylviologen concentration was 10^{-4} M in the assay and that of DCPIP 1.7×10^{-4} M. Ascorbate was given in excess and was 0.3 mM in the assay.

Preparation	O_2 Uptake [μ/mol · mg $Chl^{-1} \cdot h^{-1}$]	Chlorophyll *a/b*
N. t. var. JWB before sucrose density gradient centrifugation	340	2.5
N. t. var. JWB after sucrose density gradient centrifugation	140	4.0
N. t. Su/su after sucrose density gradient centrifugation	280	3.5
N. t. Su/su var. Aurea after sucrose density gradient centrifugation	60	3.0
Spinacia oleracea after sucrose density gradient centrifugation	400	3.2

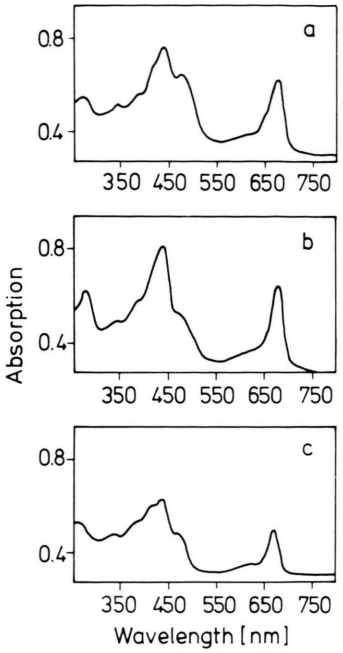

Fig. 3. Absorption spectra of the photosystem I preparations from *Nicotiana tabacum*. The concentration of preparation in the sample corresponded to 10 μg chlorophyll/ml in 0.05 M Tris buffer, pH 7.8. The measurements were carried out with a Shimadzu UV spectrometer UV-1202 over the wavelength range of 250–800 nm at room temperature. a) PS I preparation of the wild type *N. tabacum* var. JWB; b) PS I preparation from the mutant *N. tabacum* Su/su; c) PS I preparation from the mutant *N. tabacum* Su/su var. Aurea.

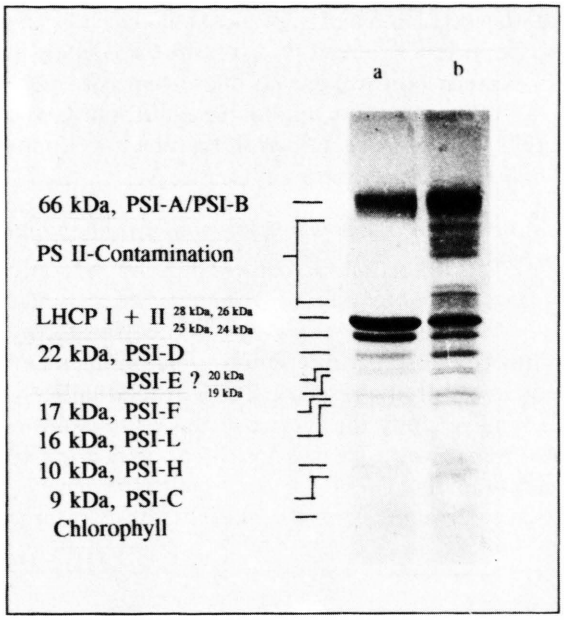

Fig. 4. Coomassie Blue-stained 10–20% SDS polyacrylamide gel showing the polypeptide composition of PS I complexes from wild type tobacco and the yellow-green mutant. a) *Nicotiana tabacum* var. JWB (8 μg chlorophyll); b) *Nicotiana tabacum* Su/su (8 μg chlorophyll).

of the preparation over the sucrose density gradient the activity decreases by more than half. The activity of the PS I preparations from the mutants is amazingly low. The absorption spectra of the PS I preparations from the three tobacco species were compared in Fig. 3. The absorption spectra exhibit pronounced absorption maxima at 438 nm and at 679 nm which corresponds to the chlorophyll *a* maxima. The spectrum of the wild type preparation is characterized by a further absorption maximum at 470 nm, which hints at a higher content of chlorophyll *b* and xanthophylls. In the spectra of the two mutants, however, the absorption in this region appears strongly reduced.

Fig. 4 summarizes the results on the analysis by SDS polyacrylamide gel electrophoresis of the peptides. The PS I preparations are composed of the core complex and the light-harvesting complex. The core complex consists of the two peptides with the apparent molecular masses 66 kDa and 9 peptides of the lesser molecular masses of 22, 20, 19, 17, 16, 10 and 9 kDa. The existence of the two low molecular weight peptides with the molecular masses of 4 and 1.5 kDa (Scheller and Møller, 1990) could not be demonstrated under the electrophoretic conditions used. The two core peptides CP1 with the apparent molecular mass of 66 kDa appear as a single protein band. The light-harvesting complex consists of 4 peptides with the molecular masses of 28, 26, 25 and 24 kDa. The two PS I preparations from the mutants contain even after purification over the sucrose density gradient still traces of the core peptides D_1 and D_2 of PS II as an impurity and also traces of the two chlorophyll-carrying peptides with the molecular masses of 43 and 47 kDa (Fig. 4). A monospecific antiserum to the purified PS I preparation reacts with the homologous peptides of PS I. In comparative analyses with PS II preparations this antiserum only labelled peptides of the light-harvesting complex. Other PS II peptides were not labelled. This means, that the obtained PS I preparations did not contain impurities from PS II and

that the 66 kDa band represents exclusively the core peptide CP1. An antiserum to CP1 labels in the Western blot exclusively these two core peptides. This antiserum inhibits in chloroplasts the methylviologen reduction with the electron donor couple DCPIP/ascorbate up to 30%.

Demonstration of carotenoids bound onto peptides

For the detection of carotenoids bound onto peptides of photosystem I, the analyzed peptides of the SDS gel electrophoresis were transferred to nitrocellulose or Immobilon-P membranes and incubated with the respective antisera. Usually, for this kind of study for every carotenoid two different antisera were used. Thus, by an antiserum to β-carotene the two CP1 core peptides were labelled with high intensity, whereas the two large subunits of the light-harvesting complex with the molecular masses 28 and 26 kDa were labelled with only low intensity (Fig. 5 and 6). For this antiserum the antigen-antibody reaction on the nitrocellulose membranes was determined in dependence on the antigen concentration as well as in dependence on the antibody concentration. This analysis showed, that with an amount of antigen corresponding to 3–10 μg of chlorophyll per assay the strong labelling intensity of the assays was the same. At concentrations corresponding to 1 μg chlorophyll the identification of β-carotene was not yet possible, and at a concentration higher than 15 μg chlorophyll per analyzing unit the reaction of the 66 kDa band was weaker again (Fig. 5). The experiments concerning the antigen-antibody reaction in dependence on the amount of anti-

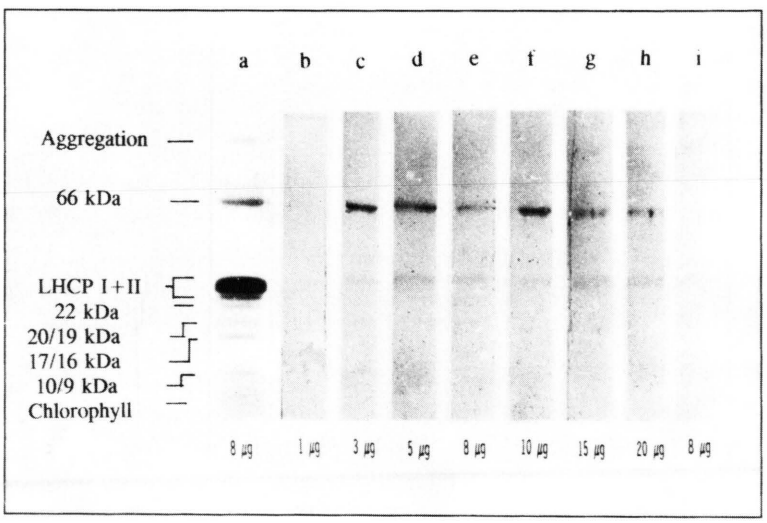

Fig. 5. Immunological determination of β-carotene by Western blot analysis of PS I polypeptides from *Nicotiana tabacum* var. JWB in dependence on the antigen concentration. a) SDS-PAGE analysis of PS I polypeptides from JWB (8 μg chlorophyll); b–i) nitrocellulose membranes with various concentrations (1–20 μg chlorophyll) of PS I preparation after the reaction with the antiserum to b–h) β-carotene (35 K_3, dilution: 1:250); i) control serum (35 K_0, dilution: 1:250).

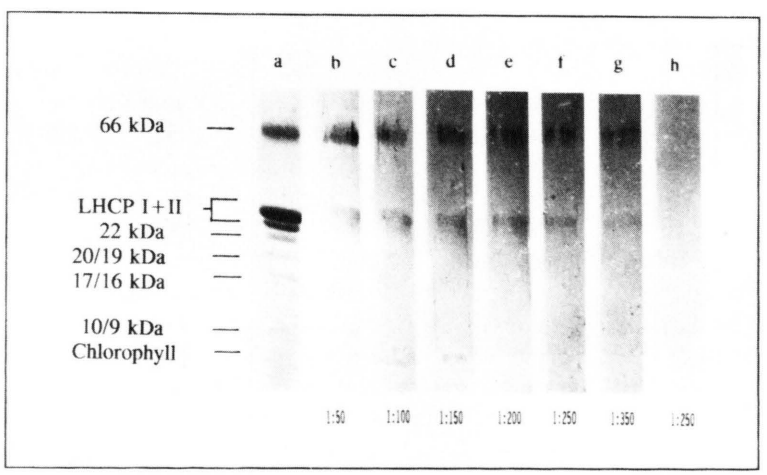

Fig. 6. Immunological determination of β-carotene by Western blot analysis of PS I polypeptides from *Nicotiana tabacum* var. JWB in dependence on the antibody concentration. a) SDS-PAGE analysis of PS I polypeptide from JWB (8 μg chlorophyll); b–h) nitrocellulose membranes with PS I polypeptides after the reaction with the b–g) β-carotene antiserum (35 K_3) of different dilutions (1:50–1:350); h) control serum (35 K_0, dilution: 1–250).

bodies used also led to the result that no sharp maximum is observed for the reaction. With an antiserum dilution between the factors 1:50 to 1:250 peptide labelling occurred with the same intensity and it was only with a dilution factor of 1:350 that the intensity of the labelling decreased again. Apparently, only a high antibody concentration leads to steric hindrance of the antibodies and to an inhibition of the binding reaction. With studies concerning the dependence of the antigen-antibody reaction on the amount of antigen on the nitrocellulose membrane it should be borne in mind that by the antigen transfer from the polyacrylamide layer to the nitrocellulose layer not all proteins/peptides are really transferred after 20 h of diffusion and that the different peptides transferred do not follow a proportional time course. Thus, a densitometric determination of the polyacrylamide gel after the transfer of the analyzed peptides and their staining showed that if an antigen amount of 6 μg protein in comparison to an amount of 37 μg is used, the amount of protein remaining in the polyacrylamide gel after the transfer does not correspond to the ratio of 1:6 but rather to 1:3. This means that using a low amount of protein up to a 2-fold higher amount of protein remains in the polyacrylamide gel. Thus, the lower the amount of antigen is, the more antigen remains in the polyacrylamide layer, and correspondingly the higher the concentration of antigen is, the higher is the transfer into the nitrocellulose membrane. The labelling experiments clearly show that β-carotene molecules are bound onto the CP1 core peptides and onto the 28 and 26 kDa peptides of the light-harvesting complex. The lower labelling of the light-harvesting molecules, however, does not necessarily mean that β-carotene molecules occur on the LHCP complex in a lesser concentration. The observation might be also due to a different carotenoid-protein binding condition and thereby be due to a different accessibility of carotene molecules to antibodies.

In Fig. 7 the reaction of the lutein and violaxanthin antisera is shown. Both antisera act differently. The antiserum to lutein labels with an exceptionally high intensity the two CP1 peptides. With these antisera two distinctly separated bands appear which permits the conclusion that both peptides contain bound lutein molecules. In contrast to this the antisera to violaxanthin label only the large subunits of the light-harvesting complex. Due to the small size of the light-harvesting complex in the Su/su mutant preparations the reaction is weaker than with the light-harvesting complex of the wild type. The two antisera to neoxanthin tested label the core peptide band as well as those of the large subunits of the light-harvesting complex (Fig. 8). However, both antisera act differently. Whereas one of the antisera strongly labels the core peptides, the other antiserum reacts in comparison stronger with the 28 and 26 kDa band of the LHCP. There are no differences of labelling intensity between the core peptides and the peptides of the LHCP from the wild type and the Su/su mutant with the neoxanthin antiserum. The corresponding control sera belonging to the antisera to β-carotene or the xanthophylls show absolutely no reaction with the described peptide bands. In Fig. 9 the reaction of the carotenoid antisera with the photosystem I peptides from the

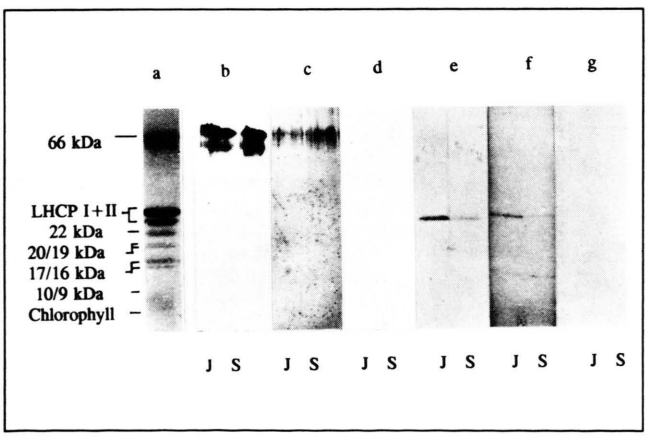

Fig. 7. Immunological determination of lutein and violaxanthin by Western blot analysis of PS I polypeptides from *Nicotiana tabacum* var. JWB (J) and the yellow-green mutant Su/su (S). SDS-PAGE analysis of PS I polypeptides from JWB (8 μg chlorophyll); b–g) nitrocellulose membranes with PS I polypeptides after the reaction with b) the lutein antiserum mixture (18/19 Ct_2, dilution: 1:100); c) the lutein antiserum mixture (21 $Ct_{1/7}$/22 $Ct_{2/4/7}$, dilution: 1:100); e) violaxanthin antiserum (14 H_8, dilution: 1:100); f) violaxanthin antiserum (45 $H_{1/2/5}$, dilution: 1:100); d) and g) control sera (serum dilutions as indicated for the respective antisera).

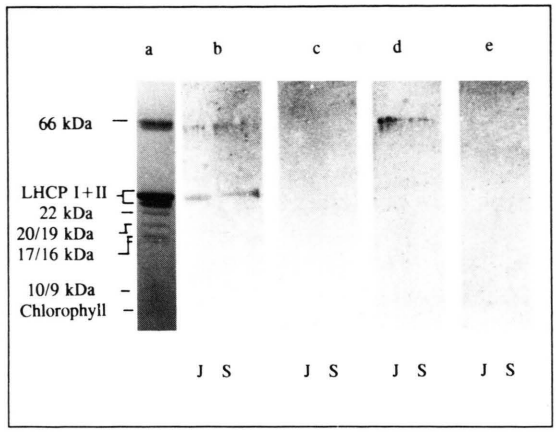

Fig. 8. Immunological determination of neoxanthin by Western blot analysis of PS I polypeptides from *Nicotiana tabacum* var. JWB (J) and the yellow-green mutant Su/su (S). a) SDS-PAGE analysis of PS I polypeptides from JWB (8 μg chlorophyll); b–e) nitrocellulose membranes with PS I polypeptides after the reaction with b) the neoxanthin antiserum (12 H_3, dilution: 1:200); d) neoxanthin antiserum (8 H_2, dilution: 1:250); c) and e) control sera (serum dilution as indicated for the respective antisera).

Aurea mutant *N. tabacum* Su/su var. Aurea is shown. Chloroplasts of this mutant differ from those of the wild type structurally. Their lamellar system has no high stacked grana but mostly single-stranded lamellae or stroma lamellae with occasional doublings. The light-harvesting complex in this mutant is small. According to the low concentration of light-harvesting complex peptides present, these peptides appear not labelled neither by the antiserum to β-carotene nor by that to neoxanthin. These two antisera react as the antiserum to lutein only with the CP1 peptides from the aurea mutant. Only the antisera to violaxanthin label the LHCP peptides with a relatively high intensity.

As the major portion of the carotenoids occurs after the SDS treatment or in the SDS gel electrophoretic analysis as soluble pigment, we could not exclude by our experiments that a translocation of these "soluble" carotenoids took place during the SDS polyacrylamide gel electrophoretic analysis. Therefore, in order to secure our results, the following two experiments on the distribution and binding of carotenoids onto peptides of photosystem I were carried out: In one experiment the PS I fractions to be analyzed were washed with acetone in order to remove "soluble" carotenoids from the preparation prior to the above described analysis of the peptides for carotenoid binding. The ensuing labelling experiments came out positively. The CP1 band as well as the LHCP peptides were labelled with the same intensity. In a second experiment the Immobilon-P membranes were washed for 30 min with methanol after the transfer of the peptides and prior to the incubation with the antisera. Also in this case the positive labelling of the peptides with carotenoid antisera was preserved which means that translocation does not occur and that the labelled carotenoids are tightly bound to the respective peptide.

The localization of lutein on the peptides of the LHCP complex is unsatisfactory with these immunological methods. As chemical analyses have clearly shown that lutein is present in LHCP lipids

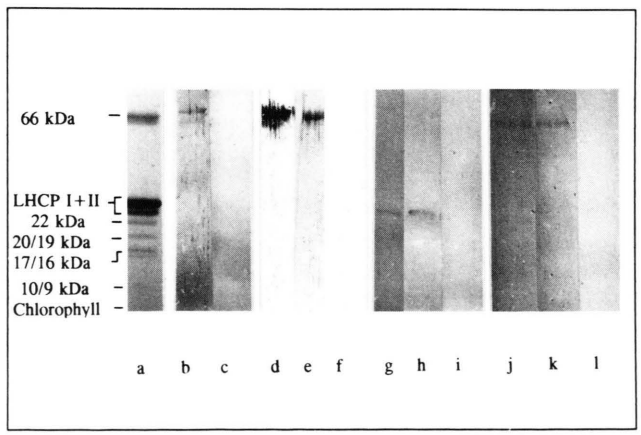

Fig. 9. Immunological determination of β-carotene, lutein, violaxanthin and neoxanthin by Western blot analysis of PS I polypeptides from the yellow mutant *Nicotiana tabacum* Su/su var. Aurea. a) SDS-PAGE analysis of PS I polypeptides from JWB (8 μg chlorophyll); b–l) nitrocellulose membranes with PS I polypeptides after the reaction with b) β-carotene antiserum (35 K_3, dilution: 1:250); d) lutein antiserum mixture (18/19 Ct_2, dilution: 1:100); e) lutein antiserum mixture (21 $Ct_{1/7}$/22 $Ct_{2/4/7}$, dilution: 1:100); g) violaxanthin antiserum (14 H_8, dilution: 1:100); h) violaxanthin antiserum (45 $H_{1/2/5}$, dilution: 1:100); j) neoxanthin antiserum (12 H_3, dilution: 1:200); k) neoxanthin antiserum (8 H_2, dilution: 1:250); c), f), i) and l) control sera (serum dilutions as indicated for the respective antisera).

in comparison to lipids from native chloroplasts in a 3-fold higher concentration (Gasser, 1993) and as the immunological test comes out negative, the accessibility of the bound lutein to antibodies seems to be the decisive problem. In order to analyze the accessibility of the carotenoids in a more general way, the carotenoid-peptide complexes were washed after the transfer to the Immobilon-P membranes during 30 min with 50 mM McIlvaine buffer, pH 3. As seen in Fig. 10, the labelling intensity of the LHCP peptides, as well as that of the CP 1 peptides by the antiserum to β-carotene decreases drastically. The same result is obtained in the labelling experiments with the lutein antiserum. However, with the antisera to violaxanthin and neoxanthin the treatment with this strongly acid buffer has not much influence on the labelling intensity. It looks as if the xanthophylls violaxanthin and neoxanthin were better protected between the protein subunits than lutein and β-carotene. As we know that the 4 carotenoids react *via* the ionon rings as the antigenic determinants with the antibodies, these molecule regions should be equally accessible to the respective antibodies. This could mean that for the structural arrangement of the carotenoids, β-carotene and lutein on the one side and violaxanthin and neoxanthin on the other, not too many differences should be possible. In order to modify or destroy the antigenic determinants of the carotenoids in these tests, the Immobilon-P membranes with the transferred peptides were exposed to a 16 h illumination at 8000 lux and subsequently incubated with the antiserum to violaxanthin. In this case the labelling is much more intensive than in the parallel test without illumination. Hence, the ionon rings of at least violaxanthin were not modified by this illumination but the structure of the protein apparently was.

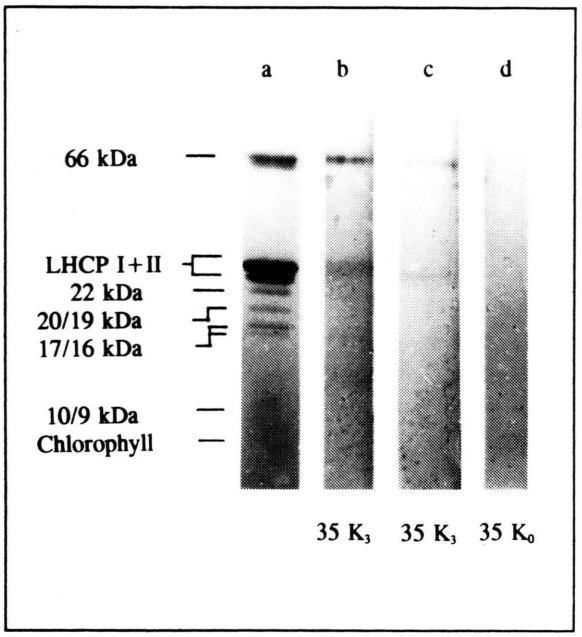

Fig. 10. Immunological determination of β-carotene by Western blot analysis of PS I polypeptides from *Nicotiana tabacum* var. JWB after incubation of the nitrocellulose membranes in McIlvaine buffer (pH 3) for 1 h. a) SDS-PAGE analysis of PS I polypeptides from JWB (8 μg chlorophyll); b–d) nitrocellulose membranes with PS I polypeptides after reaction with β-carotene antiserum (35 K_3, dilution: 1:250) and control serum (35 K_0, dilution: 1:250); b) without incubation with McIlvaine buffer (pH 3); c) with incubation with McIlvaine buffer (pH 3); d) control serum.

Discussion

Immunological test reactions with monospecific polyclonal antisera have led to the result that binding of hydrophobic substances, such as carotenoids, onto proteins can be demonstrated for the peptides of the photosystem I complex. In the case of β-carotene we were able to show that this carotenoid occurs with high concentration on the CP 1 peptides and comparatively to a lesser extent on the LHCP peptides. As under the used electrophoretic conditions the two CP 1 peptides do not migrate as separate bands, the analyses carried out, do not permit to decide whether β-carotene is bound onto both peptides or only to one of them. The result also fits into observations of the literature. Until now chemical and spectroscopic studies have shown that β-carotene is bound to the core complex of photosystem II which is composed of the two core peptides D_1 and D_2 and cytochrome b_{559} (Nanba and Satoh, 1987; Barber *et al.*, 1987; Kobayashi *et al.*, 1989; Shuvalov *et al.*, 1989; Montoya *et al.*, 1991; Braun *et al.*, 1990). In a recent publication we were able to demonstrate that β-carotene is only bound to the D_1 peptide in the filamentous cyanobacterium *Oscillatoria chalybea* (Kruse *et al.*, 1993). For the xanthophylls the used method of Western blotting has led to results which only partially fit into those of the literature. On the other hand, our re-

sults on the distribution of xanthophylls and the demonstration that violaxanthin and neoxanthin are bound to peptides of the LHCP are in agreement with the current knowledge. Absolutely new is the identification of lutein and neoxanthin on the CP1 core peptides. An unspecific adsorption or a translocation of carotenoids can, according to the carried out washing experiments of the transfer membranes with methanol, be excluded. Otherwise, in the case of unspecific adsorption one should have expected that practically all occurring peptides should be more or less labelled with all carotenoid antisera.

The test reactions clearly show that the carotenoid-antibody reactions depend on the amount of antigen as well as on the amount of antibodies used. The analyses do not give information on the molar ratios of the bound carotenoid molecules to the peptide carrier, nor do they give the information on the mode of binding. Due to the fact that the carotenoid-protein complexes survive SDS incubation and SDS gel electrophoresis, one can assume that the binding is covalent or a similarly tight-binding mode. We assume that binding occurs *via* one of the ionon rings, with the second ionon ring reaching out and being accessible to antibodies. This idea is based on the fact that carotenoid antibodies which are directed towards ionon rings positively react with the carotenoid-protein complexes.

The experiments which have been carried out in order to destroy or partially modify the carotenoid molecules as antigenic determinants did not lead to any result. Neither washing experiments with strongly acid buffers nor illumination of the transferred peptides modified the antigenic determinants in such a way that the antibody reaction was impaired. Both experiments, the acid treatment as well as the strong illumination, led simply to an isomerization of the *cis*- or *trans*-configuration. However, this configurational change did not lead to a concomitant modification of the reaction with the violaxanthin antiserum and at best only to a slight modification of the reaction with the antiserum to β-carotene and that to lutein. In further studies with the aim to detect carotenoids as antigens, tests should be made in which the carotenoids are split off the protein molecules without denaturing the proteins. Hence, enzymic cleaving reactions will be tried.

If, when using different antisera to the same carotenoid such as to lutein or to neoxanthin, the peptides of the different complexes are labelled with different intensities, giving in the extreme even no reaction at all, the interpretation must be the following: First, the respective reaction is obtained with monospecific polyclonal antibodies meaning that antibodies with different determinant specificities and thereby different binding affinity have reacted. This in turn means, that the number of the carotenoid molecules on the respective peptides must be different or that the mode of binding of the carotenoids on the peptides of the LHCP complex and the core peptides CP1 is different. As the function of the carotenoids bound to the LHCP complex or to the reaction center is obviously different (absorption of light energy and transfer of energy to the chlorophylls and probably protective functions against reactive oxygen species in the reaction center), differences in the number of bound carotenoids or differences in the binding mode and therefore different accessibilities of carotenoids to antibodies in the different functional complexes seem plausible. During the past five years comparative spectroscopic studies of the functional complexes in the purple bacterium *Rhodobacter spheroides* have shown that the carotenoids of the reaction center and the carotenoids of the LHCP complexes differ by *cis*- or *trans*-configuration (Koyama, 1993). Whereas the xanthophylls of the reaction centers have 15-*cis*-configuration, the xanthophylls bound to the LHCP complex are characterized by their all-*trans*-configuration. Furthermore, the carotenoids of the LHCP complex can occur in two different spatial arrangements, namely in a spiralic and in a planar form. According to the possibility of differing *cis-trans*-configuration and also spatial arrangement, carotenoids in the respective functional complexes will necessarily have differing accessibilities of their free ionon rings to antibodies. On the other hand, due to new physical measurements on carotenoid lipid vesicles (seminar held by Dr. W. J. Gruszecki from the Institute of Physics, Marie-Cyrie Sklodowska University, Lublin, Poland at the Chair of Cellular Physiology of the Faculty of Biology of the University of Bielefeld, Germany) and due to the dimension of the carotenoid molecules and their bipolar structure, the conclusion is reached that

carotenoid molecules might span the membrane and the ionon rings of the carotenoids would be localized in the space directed towards the stroma, as well as towards the hydrophilic region directed towards the lumen of the bipolar lipid membrane. At any rate, this would explain the accessibility of the hydrophobic carotenoid molecules also at the surface of the thylakoid membrane directed towards the stroma (Radunz and Schmid, 1973, 1975, 1979; Lehmann-Kirk *et al.*, 1979a, b; Radunz and Bader, 1982). Difficulties are encountered with the detection of lutein on the LHCP complex. By two different lutein antisera the peptides of the LHCP complex transferred to Immobilon-P membranes are, if at all, only slightly labelled. However, with chemical methods we were able to demonstrate unequivocally that the LHCP complex of *Nicotiana tabacum* (Gasser, 1993) as well as the LHCP of spinach (Ryrie *et al.*, 1980) contain lutein in high concentration. If the Western blot procedure nevertheless yields no labelling, it must be concluded, that the main portion of lutein is only adsorbed to the complex and removed by the SDS treatment and SDS gel electrophoresis or that bound lutein molecules are not accessible to antibodies. Finally, it must be borne in mind, that the SDS gel chromatographic analysis might lead to a partial denaturing of the proteins representing in fact conformational changes. These conformational changes might lead to a change in orientation or to a displacement of carotenoid molecules. (It should be pointed out that the reaction of the lutein antisera with peptides of the LHCP complex from PS II came out positively.) In contrast to this, violaxanthin was found to be bound only to peptides of the light-harvesting complex.

With the demonstration of carotenoids as chromophores on the core peptides and on the peptides of the LHCP complex of photosystem I our earlier observations on the inhibitory effect of antisera to carotenoids on photosynthetic electron transport on the donor side of photosystem I finally find a plausible explanation (Radunz and Schmid, 1973, 1975, 1979; Lehmann-Kirk *et al.*, 1979a, b; Radunz and Bader, 1982). Our earlier concept that carotenoids must be bound as chromophores onto the peptides involved in photosynthetic electron transport finds its confirmation.

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