The Effect of Antibodies to Violaxanthin on Photosynthetic Electron Transport

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An antiserum to violaxanthin inhibits photosynthetic electron transport between water, iodide or tetramethylbenzidine and various electron acceptors in chloroplasts from green tobacco *(Nicotiana tabacum var. John William's Broadleaf)*. However, electron transport from manganese or diphenylcarbazide to these acceptors is not impaired. The typical photosystem I reaction from DPIP/ascorbate to anthraquinone-2-sulfonate in the presence of DCMU shows no inhibition. From this it is concluded that the effect of violaxanthin on the photosynthetic electron transport chain lies on the oxygen-evolving side of photosystem II before the site from which diphenylcarbazide or manganese donate electrons.

In the presence of DCMU after preillumination we find an effect of the antiserum on fluorescence.

The reaction of the antibodies to violaxanthin with stroma-freed chloroplasts depends on the condition of the thylakoid membrane. Chloroplasts which are still swellable react in a bivalent manner and are agglutinated. Non swellable chloroplasts react only in a monovalent manner. This specific binding was demonstrated by means of the Coombs-test.

From these reactions it follows that the antigenic determinants of violaxanthin are accessible to the antibodies, hence, they must be located in the outer surface of the thylakoid membrane.

In earlier publications we have reported on the properties of antisera to the carotenoids lutein and neoxanthin [1, 2]. It appeared that these antisera inhibited photosynthetic electron transport on the oxygen-evolving side of photosystem II. However, the degree of inhibition on photosynthetic electron transport with these two antisera was rather low namely 10–20% which was interpreted as being due to the fact that only part of these carotenoids somehow involved in a photosystem II reaction, were accessible to antibodies in the thylakoid membrane. This could have been the case if the carotenoids are either mainly located inside the membrane or in partition regions of the grana but nevertheless in the outside face of the membrane. This way antibodies would have no access to the antigen. Investigations on the maximal binding of antibodies onto the outer surface of the thylakoid membrane showed, that only every 24th molecule of lutein and every 10th molecule of neoxanthin binds one antibody molecule [3, 4]. The idea of the carotenoids being located in the partition regions was later substantiated by the observation of Schmid, List, and Radunz who observed that thylakoid preparations of the bluegreen algae *Nostoc muscorum* or *Oscillatoria chalybea* were inhibited by 50–60% with respect to their photosynthetic electron transport capacity [5]. As is known from the literature the lamellar system of these bluegreen algae consists of single unfolded thylakoids [6].

In the following we report on the properties of an antiserum to violaxanthin. This antiserum inhibits photosynthetic electron transport in tobacco chloroplasts consistently to a higher degree than the earlier described antisera. Again, the inhibition site is located on the oxygen evolving side of photosystem II.

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Abbreviations: DPIP, 2,6-dichlorophenol-indophenol; DCMU, N,N'-3,4-dichlorophenyl-dimethylurea; DPC, diphenylcarbazide; A-2-sulf, anthraquinone-2-sulfonate; TMB, tetramethylbenzidine.

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Materials and Methods

Isolation of violaxanthin (3,3'-dihydroxy-5,6,5'6'-diepoxy-β-carotene):

Ether soluble lipids from *Urtica dioica* were saponified at room temperature with 5 per cent sodium ethylate. The carotenoids were extracted with diethyl ether and then removed by column chromato-

graphy on cellulose (MN 2100 ff – Macherey, Nagel & Co., Germany). Petrolether (hp 40 – 60 °C) and acetone (1 – 20%) were used to elute the carotenoids; first lutein, then violaxanthin and finally neoxanthin [2].

The obtained violaxanthin fraction was rechromatographed by column chromatography on aluminium oxide (aluminium oxide 60, alkaline, activity type II, Merck) and eluted with petrol ether and ethanol (absolute) by increasing concentration gradients from 0.5 – 2 per cent.

The violaxanthin fraction was finally purified by thin layer chromatography ($MgOH \times CO_3$/silicious earth (2:1)).

The UV spectrum of violaxanthin in ethanol had maxima at 470, 441 and 416 nm (Fig. 1) [7, 8].

Preparation of the antiserum:

Immunization of rabbits with violaxanthin was carried out as described earlier [1, 2].

Chloroplast preparations:

Stroma-free swellable chloroplasts from *Nicotiana tabacum* var. John William's Broadleaf and from yellow leaf patches of the variegated tobacco mutant *Nicotina tabacum* var. NC 95 were prepared according to Homann and Schmid [9]. The variegated tobacco mutant NC 95 is described in an earlier publication [10]. Stroma-freed non swellable chloroplasts from *Antirrhinum majus* were prepared according to Kreutz and Menke [11]. The preparation of thylakoids from *Oscillatoria chalybea* was carried out as described earlier [5, 12].

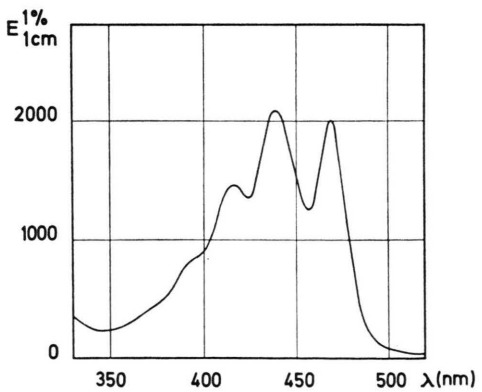

Fig. 1. Spectrum of the pure violaxanthin preparation used for injection into the rabbits.

In order to destroy the oxygen evolving capacity, the chloroplasts were washed with 0.8 M Tris pH 8 according to Yamashita and Butler [13].

Electron transport reactions were carried out essentially as in the assays described previously [12, 14].

Incubation with the serum was done in the dark for 5 minutes. Where indicated, the whole assay without acceptor was preilluminated for 5 minutes.

Silicomolybdate reduction with water as the electron donor was measured either as O_2 evolution with a Clark type electrode (Rank Broth., England), or by its direct reduction which is accompanied by an absorbance increase at 750 nm using either H_2O, iodide or manganese as electron donor. Rates of SM-reduction were calculated using a millimolar extinction coefficient of $8\ mM^{-1} \times cm^{-1}$ [15].

Sodium-silicomolybdate was synthetised according to the prescription from Jander and Blasius [16]. The obtained solution was evaporated to dryness. Where indicated, a solution of 2.5×10^{-3} g dry weight from this preparation was used per assay.

Fluorescence measurements were carried out with a modified Perkin-Elmer Fluorescence Spectrophotometer MPF-3 equipped with a Siemens oscillograph model Oscillar MO 7114.

Results

The antiserum to violaxanthin agglutinates stroma-free swellable chloroplasts from *Nicotiana tabacum* var. John William's Broadleaf, hence violaxanthin is located in the outer surface of the thylakoid membrane. The fact that violaxanthin is located in the outer surface of the thylakoid membrane is further demonstrated by the exhaustion test in which 1 ml of antiserum was incubated for 12 hours with increasing amounts of stroma-freed non swellable chloroplasts from *Antirrhinum majus*. The mixture was centrifuged and the sediment discarded. Subsequently, the supernatant was tested for the remaining inhibitory capacity against stroma-free swellable chloroplasts in a photosynthetic electron transport reaction. The obtained titration curve (Fig. 2) shows that the antiserum can be fully exhausted with chloroplasts which means that violaxanthin is surface located to an appreciable extent.

The localization of the inhibition site follows from the inhibitory action of the antiserum on the photo-

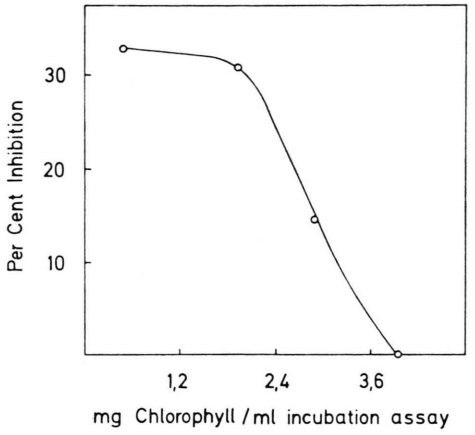

Fig. 2. Remaining inhibitory capacity of which increasing amounts of chloroplasts exhausted violaxanthin antiserum. The effect was tested in the reaction $H_2O \rightarrow$ anthraquinone-2-sulfonate.

synthetic electron transport reactions summarized in Table I. It is quite obvious from these results that the inhibition site should be located on the oxygen-evolving side of photosystem II between the sites of electron donation of water, tetramethylbenzidine or iodide on the one hand and the sites of electron donation of diphenylcarbazide or Mn^{2+} on the other hand. Typical photosystem I reactions such as the DPIP/ascorbate driven photoreduction of anthraquinone-2-sulfonate in the presence of DCMU are not inhibited. It is noteworthy that the photoreduction of silicomolybdate is only inhibited if H_2O or iodide are the donors (Table I). According to the literature silicomolybdate accepts its electrons before the DCMU-block. The reaction can be identified as a photosystem II-reaction by its heat-sensitivity [15].

The inhibition of electron transport caused by the antiserum involves a light effect. That is, in an electron transport reaction for example in the anthraquinone-2-sulfonate Mehler-type Hill reaction the inhibition develops in the course of the light reaction. It takes minutes for the effect to show fully up (Fig. 3). Therefore, all reactions with the acceptors DPIP or silicomolybdate were preilluminated, because these reactions only last about one (silicomolybdate) to a few (DPIP) minutes. This fact must be considered when discussing the somewhat lower extent of inhibition in these reactions (Table I).

The antiserum also inhibits electron transport in thylakoid preparations of the bluegreen algae *Oscillatoria chalybea*. However, the degree of inhibition

Table I. Effect of the antiserum to violaxanthin on different electron transport reactions in wild type tobacco chloroplasts.

Reaction		Rate [μmol acceptor reduced $mg \cdot Chl^{-1} \cdot h^{-1}$]	% Inhibition
$H_2O \rightarrow$ DPIP	Antiserum	218	
	Control serum	289	25
$H_2O \rightarrow SiMoO_4^{2-}$	Antiserum [b]	105	
	Control serum	157	33
$H_2O \rightarrow$ A-2-sulf	Antiserum	259	
	Control serum	378	31.36
DPC $\rightarrow$ DPIP	Antiserum [a]	101	
	Control serum	101	0
$Mn^{2+} \rightarrow SiMoO_4^{2-}$	Antiserum [a, b]	21	
	Control serum	21	0
$J^- \rightarrow SiMoO_4^{2-}$	Antiserum [a, b]	19	
	Control serum	24	20
$Mn^{2+} \rightarrow$ A-2-sulf	Antiserum [a]	570	
	Control serum	566	0
$J^- \rightarrow$ A-2-sulf	Antiserum [a]	239	
	Control serum	277	14
TMB/asc. $\rightarrow$ $\rightarrow$ A-2-sulf	Antiserum [a]	370	
	Control serum	570	31.26
DPC $\rightarrow$ A-2-sulf	Antiserum [a]	670	
	Control serum	714	0
DPIP/asc. $\rightarrow$ $\rightarrow$ A-2-sulf	Antiserum [b, c]	436	
	Control serum	454	0

[a] TRIS washed chloroplasts.
[b] Reaction not sensitive to 10^{-6} M DCMU in the assay.
[c] Reaction carried out with the variegated tobacco mutant NC 95.
All reactions with DPIP or $SiMoO_4^{2-}$ as acceptors were carried out after 5 min of preillumination.

was not superior to that reported for tobacco chloroplasts which is a difference to the antisera to lutein and neoxanthin [5]. The electron transport reactions tested were $H_2O \rightarrow$ A-2-sulf and $H_2O \rightarrow$ $\rightarrow$ DPIP which were both inhibited and DPC $\rightarrow$ A-2-sulf which was not inhibited or inhibited to a lesser extent.

The effect of the antiserum on fluorescence induction in tobacco chloroplasts shows the following: No effect of the antiserum on fluorescence in the electron transport system is seen with dark adapted or preilluminated chloroplasts. However, a clear difference is seen in the presence of DCMU after preillumination. The effect of the antiserum in the presence of DCMU consists in a lowering of the steady state level of the variable fluorescence.

Discussion

The effect of the antiserum to violaxanthin on photosynthetic electron transport reactions in tobacco chloroplasts shows again that the site of inhibition is on the oxygen-evolving side of photosystem II.

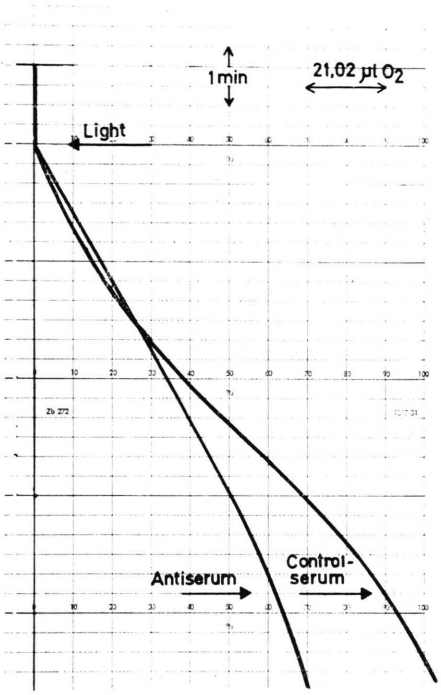

Fig. 3. Effect of the antiserum to violaxanthin on electron transport measured as oxygen uptake in an anthraquinone-2-sulfonate mediated Mehler reaction with water as the electron donor.

The difference to the earlier reported antisera to lutein [1] and neoxanthin [2] is essentially only the higher degree of inhibition induced by the antiserum to violaxanthin even though in detail a difference in the localization of the inhibition site on the oxygen-evolving side of photosystem II seems to exist also. As has been said before [1, 2] we do not believe that any of these carotenoids are directly involved as carriers in photosynthetic electron transport. However, we do believe that the antisera act in a very specific manner with the individual carotenoid to which the antisera are directed. In this sence the inhibition of the electron transport reactions must be visualised as being induced by a conformational change of a protein or polypeptide to which the respective carotenoid is attached with the carotenoid being accessible to the antibodies. This is further substantiated by the fact that an antiserum to auroxanthin, a carotenoid which does not occur in chloroplasts, and whose structure differs from that of violaxanthin only by the position of the two epoxy groups, does not inhibit electron transport.

In this context we should like to mention again that we had explained the low degree of inhibition of electron transport (10 – 20% by the antisera to lutein and neoxanthin, 30% by the antiserum to violaxanthin) by the possibility that the xanthophylls are located in partition regions of the lamellar system to which antibodies would have no access. This interpretation was substantiated by Schmid *et al.* [5] who observed that electron transport in thylakoid preparations of the bluegreen algae *Oscillatoria chalybea* and *Nostoc muscorum* was inhibited by 50 – 60% by the same antisera which inhibited tobacco chloroplatsts by only 10 – 20%. The observation fits nicely into results by Trosper and Allen [17] who showed that the carotenoid distribution in chloroplasts of plants was such that 2/3 of the xanthophylls were located in the grana regions. Moreover, the involvement of xanthophylls in the photosystem II activity of *Scenedesmus obliquus* is demonstrated by a very recent report of Straßberger and Senger [18] who show that xanthophylls are essential for the organization of photosystem II.

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