Action of an Antiserum to α-Tocoquinone on Photosystem II-Particle Preparations of *Nicotiana tabacum*

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An antiserum to α-tocoquinone was prepared by immunization of rabbits. Immunization was obtained by injection of a conjugate consisting of the hapten α-tocoquinone attached to methylated ovalbumin into the rabbit. The antiserum recognizes the 3,4-dimethyl-*p*-benzoquinone group of the molecule as well as part of the immediate vicinity to the side chain. This is concluded from the fact that the antibody has some affinity also to plastoquinone. No reaction of the antibody is observed with α-tocopherol hydroquinone or α-tocopherol. Reaction of the antiserum to α-tocoquinone with photosystem II-particle preparations from tobacco affects the functionality of the preparation. Chlorophyll$_a$-fluorescence emission is quenched without an alteration of the emission spectrum. Concomitant with this fluorescence quenching, the lifetime of two fluorescence components namely that of a *fast* and a *slower* component are shortened. By analogy with the literature the *fast* component is associated with chlorophyll$_a$ of the reaction center core and that of the *slow* component with the antenna system in which the lifetime parameter is shortened by the antibody from 3.42 ns to 1.795 ns. The action on the *fast* component is less and leads to a shortening of the lifetime parameter from 0.373 ns to only 0.249 ns. The effect is interpreted in terms of an enhancement of linear photosynthetic electron transport possibly due to an inhibition of the cyclic electron transport around PS II, discovered by Gruszecki *et al.* (1995), Z. Naturforsch. **50c,** 61–68.

Introduction

The photosynthetic apparatus of green algae and higher plants contains a series of prenylquinones. The main component is plastoquinone. Besides this compound the quinones phylloquinone (vitamin K_1), α-tocopherol and α-tocoquinone occur in lesser amounts (Kirk and Tilney-Basset, 1978; Lichtenthaler, 1977; Lichtenthaler, 1969). Plastoquinone plays an important role as proton carrier in the photosynthetic membrane and occurs as a free pool as well as in bound form as Q_A and Q_B in photosystem II (Velthuys, 1982; Amesz, 1987). The role of the other chloroplast quinones appears less clear. α-tocopherol is generally associated with a protective function of the thylakoid membrane against oxidations and phylloquinone with electron transport on the reducing side of photosystem I (Lichtenthaler, 1987). Only little is known about the properties and function of α-tocoquinone. It is known to occur in the thylakoid membrane of chloroplasts (Lichtenthaler, 1979), but only in lesser amounts in plastoglobuli (Lichtenthaler, 1987). As already stated, the function of α-tocoquinone as that of the other tocoquinone species is so far unknown. There are reports that Hill reactions are stimulated if the chloroplast membrane has been extracted with organic solvents and then reconstituted with α-tocoquinone which possibly indicates a role in photosynthetic electron transport (Bucke *et al.*, 1966; Lichtenthaler, 1980).

According to the available data only a small amount of α-tocoquinone occurs in photosystem I and photosystem II preparations (Tabata *et al.*, 1985; Schoeder and Lockau, 1986) what might hint at the possibility that the major amount of the α-tocoquinone present in the thylakoid membrane occurs as a free pool in the lipid matrix rather then

Abbreviations: PS II, photosystem II; α-TQ, α-tocoquinone.

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bound to proteins (Lichtenthaler, 1980). Recent studies on light-driven electron transport in liposome-bound photosystem II particles between water and ferricyanide showed that modification of the experimental system with the exogenous quinones α-tocoquinone or plastoquinone resulted in pronounced effects on photosynthetic oxygen evolution. Thus, the presence of α-tocoquinone in photosystem II samples decreased the rate of red light-induced oxygen evolution but enhanced green light-induced oxygen evolution (Gruszecki *et al.*, 1995). These findings were interpreted in terms of a cyclic electron transport around photosystem II. The mechanism for this electron transport is seen via the energetic coupling of β-carotene in the reaction center of photosystem II and that of antenna carotenoid pigments regulated by violaxanthin which is part of the xanthophyll cycle (Gruszecki *et al.*, 1996). In the present paper we demonstrate the preparation of a monospecific polyclonal antiserum to α-tocoquinone and describe its serological specificity as well as its action on photosystem II-particles of tobacco. The paper contributes to the understanding of the role of α-tocoquinone in the photosynthetic membrane of higher plants.

Material and Methods

Photosystem II particles were isolated according to the procedure described by Berthold *et al.* (1981) from chloroplasts of wild-type tobacco *N. tabacum* var. John William's Broadleaf and from the aurea mutant *N. tabacum* Su/su derived from it (Schmid, 1967; Homann and Schmid, 1967). These preparations were suspended in 30 mM Tricine(N-tris[hydroxyl]-methylglycine)-NaOH buffer (pH 7.5) containing 60 mM KCl.

Preparation of α-tocoquinone and preparation of the antiserum.

α-D,L-tocoquinone was obtained from α-D,L-tocopherol (Merck, Darmstadt) by oxidation with $FeCl_3$ and separated from other α-tocopherol oxidation products by column chromatography on silica gel (Merck, Darmstadt) with the solvent benzene:acetone:heptane (96:4:2, v/v/v). The purity of α-tocoquinone was verified by measurement of the absorption spectrum in the ultraviolet range. As purity criterium we used the absorbance ratio between the maximum of the spectrum at 262 nm and the minimum at 220 nm in absolute ethanol. The preparation used had the absorbance ratio 6.2 and was considered absolutely pure. Preparation of the antiserum to α-tocoquinone was achieved according to the hapten-principle described by Schmid *et al.* (1993) for the preparation of antibodies to lipids. The porter-molecule used was methylated ovalbumin (albumin from chicken, fraction V powder, Sigma). Methylated ovalbumin is a basic protein onto which a multitude of lipids can be attached via ionic bounds. For the binding 2 mg absolutely pure α-tocoquinone were suspended with 1 mg methylated ovalbumin in 1 ml phosphate buffer (0.06 M Na_2HPO_4/KH_2PO_4, pH 7.4) emulgated with 1 ml complete Freund's adjuvant and injected subcutaneously into a rabbit. After 24 days we made 5 booster injections at 2 days intervals injecting each time the same amount of α-tocoquinone intravenously without the adjuvant. 8 days after the last booster injection 10 ml blood were withdrawn from the animal followed by further 10 ml blood withdrawals after every week until the titer of the antiserum became too low for serological investigations. For comparison an antiserum to α-tocopherol was prepared according to the same procedure. The titer of the antiserum and the specificity were determined according to the dilution methods in the ELISA-Test (see Fig. 1). Control sera obtained from the respective animals before immunization gave no effect.

Fluorescence lifetime kinetics of chlorophyll were measured using a K_2-Multifrequency Cross-Correlation Phase and Modulation Fluorometer (ISS, Urbana-Champaign, Illinois, USA) equipped with a xenon lamp and a Pockels cell light modulator. A diluted solution of glycogen was used as a light scattering reference. For excitation 440 nm monochromatic light modulated at frequencies up to 250 Mhz was applied (Lakowicz, 1983).

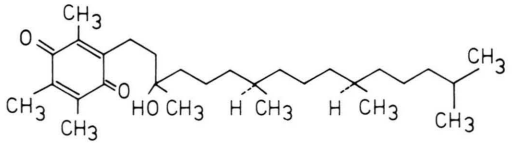
Results and Discussion

a) Monospecificity of the antiserum to α-tocoquinone

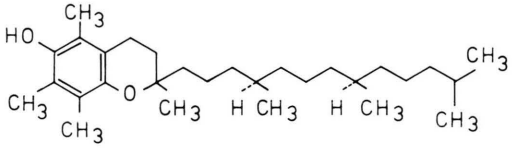
The activity and specificity of the obtained antiserum to α-tocoquinone was tested in the Elisa-dilution test (Schmid *et al.*, 1993) with the homologous antigen α-tocoquinone and the two non-ho-

mologous antigens α-tocopherol and plastoquinone at the antigen concentrations given in Fig. 1, ranging from 600 to 2400 ng and serum dilutions ranging from 1/25 to 1/1000, respectively to 1/6000 (v/v). As seen from Fig. 1, the antiserum to α-tocoquinone gives with the homologous antigen the highest yield of 4-nitrophenolate, resulting from the action of the alcaline phosphatase bound to the second antibody. This yield corresponds with an antigen concentration of 1200 ng and a serum dilution of 1:50 to 57 nmol 4-nitrophenolate (Fig. 1a). With the non-homologous antigen α-tocopherol the reaction yields only 1/5 of the amount (Fig. 1b). Only plastoquinone with an antigen concentration of 2400 ng and a serum dilution of 1:50 leads to a comparable yield of 39 nmol 4-nitrophenolate.

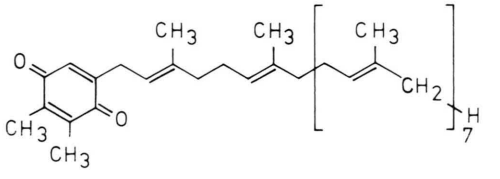
A comparison of the chemical formulas of the three components (Scheme) shows that the antibody is directed towards the quinone ring which explains the reaction with plastoquinone and the non-reaction with α-tocopherol, since the antibody apparently does not recognize the quinol function. Somehow the immediate vicinity of the quinone ring to the terpenoid chain must be included or play a role in the antibody specificity otherwise the smaller affinity towards plastoquinone cannot be explained.



α-Tocoquinone



α-Tocopherol



Plastoquinone

b) Fluorescence emission and fluorescence lifetime of chlorophyll in photosystem II particles affected by the antiserum to α-tocoquinone (α-TQ)

The antiserum to α-TQ had a distinct effect on fluorescence properties of the PS II-particles. The effect was exerted on both fluorescence emission intensities and the lifetime of the fluorescence components. Thus, the antiserum to α-TQ quenches fluorescence emission of the PS II-particles without changing an apparent feature of the fluorescence emission spectrum (Fig. 2a). The experiment has been carried out in the presence of the antiserum to α-TQ and for a control in the presence of control serum which is serum withdrawn from the animal before immunization (Fig. 2a). The effect is considerable. The difference between the antiserum and control serum represents an effect which is comparable, in these particles, to the one inflicted by 10^{-5} M DCMU (3(3,4-dichlorophenyl)-1,1-dimethylurea) (Fig. 2b). It should be emphasized, however, that DCMU enhances fluorescence emission, whereas the antiserum to α-TQ quenches it (Fig. 2b). If fluorescence emission is interpreted in terms of an emanation of electron transport efficiency, the effect of the antiserum would mean stimulation of electron transport. As α-tocoquinone might inhibit or regulate plastoquinone reduction, we tested the antiserum after addition of plastoquinone. Apparently plastoquinone addition did not modify the action of the antiserum (experiments not shown). The antiserum exerts, however, an even bigger role on fluorescence emission of PS II-particles prepared from the aurea mutant Su/su (Fig. 2c). The mutant which efficiently accumulates zeaxanthin from violaxanthin by photoconversion and by *de novo* biosynthesis is known to exhibit a peculiar fluorescence emission behaviour (Schindler and Lichtenthaler, 1994). The effect is very pronounced and as the mutant is particularly well known for its reduced photosynthetic unit (Schmid, 1967; Homann and Schmid, 1967; Okabe *et al.*,1977; Canaani *et al.*,1985), the interpretation of the function of α-TQ might lie in the regulation of electron transport mediated by the reaction center via the light absorbing pigment system.

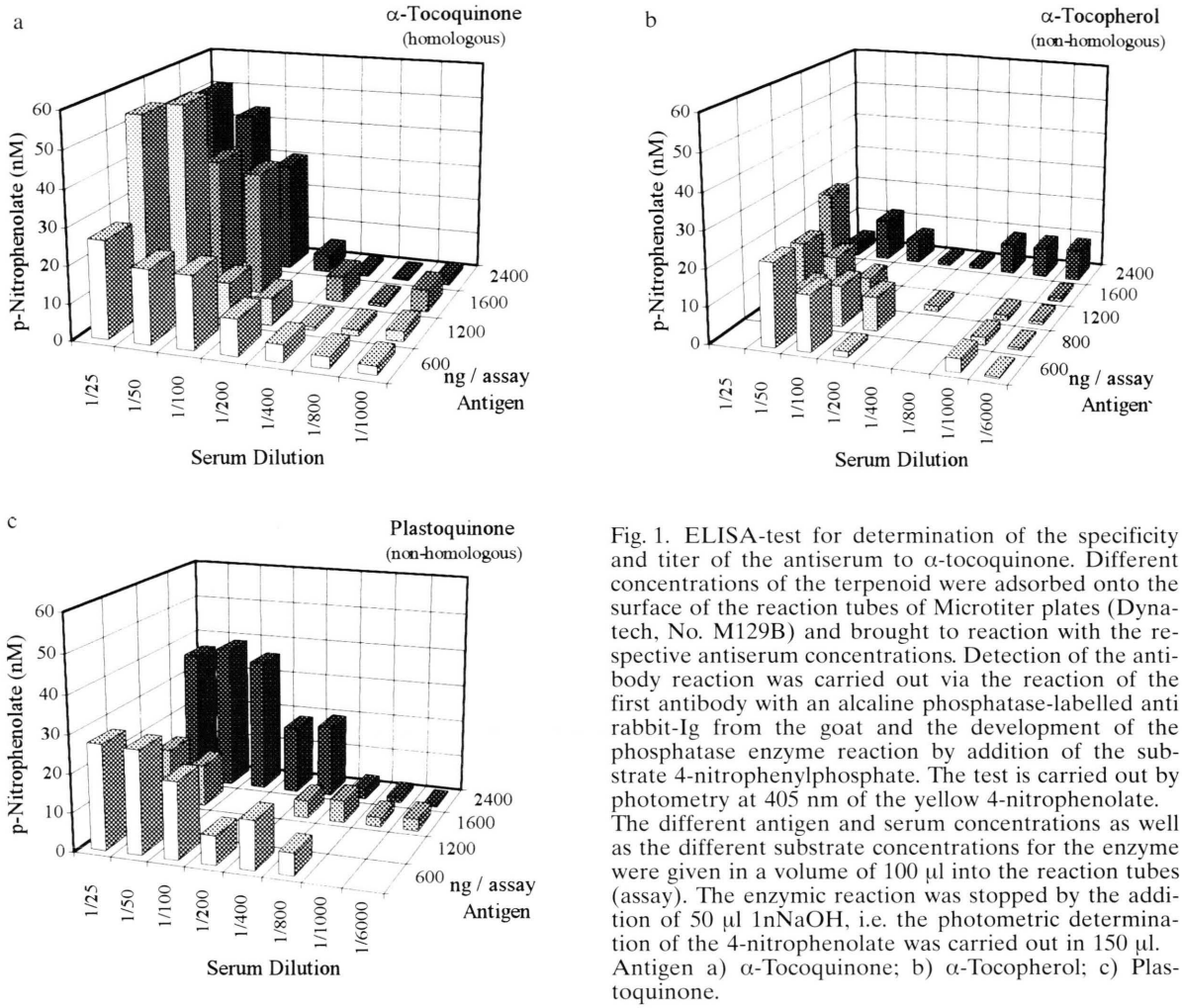


Fig. 1. ELISA-test for determination of the specificity and titer of the antiserum to α-tocoquinone. Different concentrations of the terpenoid were adsorbed onto the surface of the reaction tubes of Microtiter plates (Dynatech, No. M129B) and brought to reaction with the respective antiserum concentrations. Detection of the antibody reaction was carried out via the reaction of the first antibody with an alcaline phosphatase-labelled anti rabbit-Ig from the goat and the development of the phosphatase enzyme reaction by addition of the substrate 4-nitrophenylphosphate. The test is carried out by photometry at 405 nm of the yellow 4-nitrophenolate. The different antigen and serum concentrations as well as the different substrate concentrations for the enzyme were given in a volume of 100 µl into the reaction tubes (assay). The enzymic reaction was stopped by the addition of 50 µl 1nNaOH, i.e. the photometric determination of the 4-nitrophenolate was carried out in 150 µl. Antigen a) α-Tocoquinone; b) α-Tocopherol; c) Plastoquinone.

In order to further analyze the action of the antiserum to α-tocoquinone we analyzed the fluorescence lifetime (Table I). Three components were fitted into the experimental fluorescence decay curves, designated as *fast*, *middle* and *slow*. Only two components, the *fast* and *middle* component are further analyzed, as the third *slow* component shows only a negligible amplitude (i.e. has a low

Table I. Effect of the antiserum to α-tocoquinone on the fluorescence lifetime of chlorophyll in photosystem II-particle preparations of *Nicotiana tabacum*.

				Fluorescence component			
Sera	Lifetime τ [ns]			Amplitude/abundance (normalized)			Square of standard deviation ($æ^2$)
	fast	*middle*	*slow*	*fast*	*middle*	*slow*	
Antiserum to α-tocoquinone	0.249	1.795	75.478	0.822	0.159	0.018	6.7
control serum	0.373	3.142	284.72	0.888	0.100	0.012	6.8

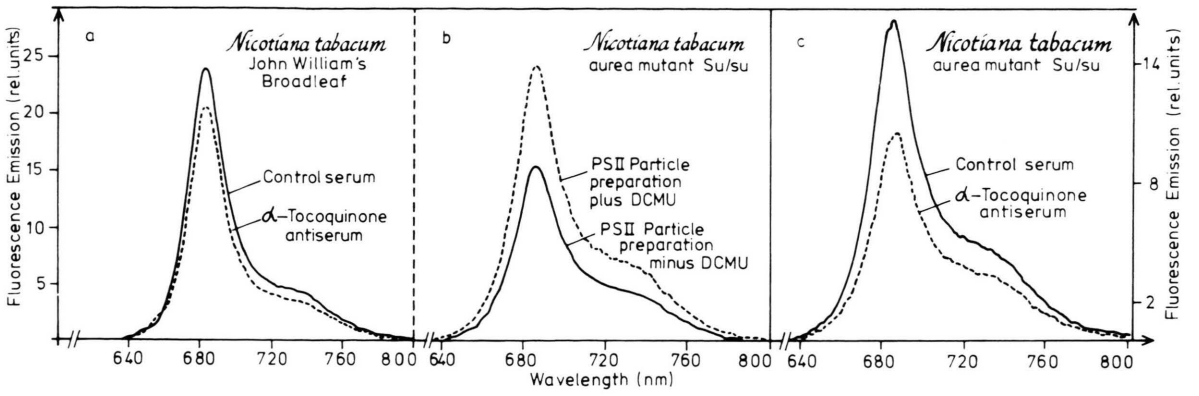


Fig. 2. **a.** Fluorescence emission spectrum of photosystem II particle preparations *of Nicotiana tabacum* var. John William's Broadleaf (wild-type) in the presence of the antiserum to α-tocoquinone (----) and in the presence of control serum (—). Chlorophyll concentration per 2 ml assay (30 mM Tricine-NaOH buffer, pH 7.5, containing 60 mM KCl), 40 μg. Excitation wavelength 440 nm.
b. Fluorescence emission spectrum of photosystem II particle preparations of the aurea mutant *Nicotiana tabacum* Su/su in the presence of 10^{-5} M DCMU (---). Control assay without DCMU (—). Chlorophyll concentration per 2 ml assay (30 mM Tricine-NaOH buffer, pH 7.5, containing 60 mM KCl) 40 μg.
c. Fluorescence emission spectrum of photosystem II particle preparations of the aurea mutant *Nicotiana tabacum* Su/su in the presence of the antiserum to α-tocoquinone (---) and in the presence of the control serum. (—) Chlorophyll concentration and excitation wavelength as in **b**.

abundance and should in all probability be impurity-related). Thus, in PS II-particles treated with the control serum the lifetime of the *fast* component (τ_f) is 0.373 ns and its amplitude/abundance in the normalized system 0.888 (88.8%), making it the dominant component in the system. The lifetime of the *middle* component (τ_m) has a value of 3.142 ns with a much lower amplitude of 0.100 hence an abundance of 10% (Table I). Treatment of PS II-particles with anti α-TQ serum had a distinct effect on these two components. It lowered the τ_f-value to 0.279 ns and that of τ_m to 1.795 ns. Important is that the amplitude of the *fast* component is lowered whereas that of the *middle* component is increased. The main effect concerning both parameters lies on the *middle* component. Here, the fluorescence lifetime is lowered by nearly one half and the amplitude is increased by approx. 50% (Table I) whereas the effect of the antiserum on the amplitude of the *fast* component is lesser.

At first glance, the observed effect as seen from the specificity of antiserum (Fig. 1) might be related to plastoquinone and not to α-tocoquinone. However, an earlier described antiserum to plastoquinone inhibited electron transport and concomitantly enhanced fluorescence (Radunz and Schmid, 1973). In the present paper, the antiserum stimulated electron transport and quenched fluorescence with a major effect on a chlorophyll component which is generally associated with the light antenna.

The observed changes in the fluorescence properties are to be interpreted in general terms of competitive processes of the photochemical electron transfer, dissipation of energy via fluorescence or other deactivation pathways. In our system we certainly do not observe any fluorescence emission from free chlorophyll coupled energetically with the antenna system. The lifetime of this type of fluorescence emission would lie typically around 5 ns. Also, it is not possible to ascribe unequivocally the origin of the *fast* and the *middle* component in our system, since various factors may have an impact on the measured lifetimes, such as open or closed reaction centers (Karukstis and Sauer, 1983), the actual composition of the PS II-particles and the used plant species. Tentatively, by analogy to greening and other green systems (Myśliwa-Kurdziel *et al.*, 1996) we dare to attribute the *fast* component to the chlorophylls of the reaction center core, and the *middle* component to the antenna system. As pointed out many years ago (for review and reference see Trebst and Avron, 1977) and as confirmed by Schatz *et al.* (1988),

large antenna systems are at the origin of increased fluorescence time constants, due to the random walk of excitons in the pigment bed.

As already stated above, the effect of the antiserum to α-TQ on steady state fluorescence emission and the fluorescence lifetime might be interpreted in terms of an increased electron transport. Generally, one might think according to the principle that less fluorescence means less lifetime. However, one should bear in mind that a shortening of fluorescence lifetime may not necessarily be interpreted in terms of an activation of electron flow. This is seen from the fact that the lifetime of chlorophyll fluorescence in isolated LHCP II, where no photochemistry occurs, is around 1.2 ns due to an increased non-radiative decay (Nordlung and Knox, 1981; Il'ina *et al.*, 1981). Stimulation of linear electron transport would also occur if the cyclic electron flow around photosystem II reported by Gruszecki *et al.*, (1995 and 1996) would be inhibited (by the antibody binding). In this case the question arises whether α-TQ is indeed a component of this cyclic electron transport as postulated by these authors.

A definite conclusion is not yet possible. Presumably α-TQ participates in the photosynthetic electron transport chain (Kruk and Strzałka, 1995). One may speculate that the observed effect may be attributed either to a direct or indirect enhancement of photosynthetic electron flow caused by the antibody binding or to a reorganization of antenna complexes and changes in energetic coupling between chlorophyll molecules. The answer to these questions will be the subject of future studies.

Acknowledgements

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