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Red-light-induced photoreactions of chlorophyll a mixtures with all-*trans*- or 9-*cis*-β-carotene

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Abstract

The protection of chlorophyll a from red light ($\lambda > 630$ nm) induced photobleaching was compared for 9-*cis*- and all-*trans*-β-carotene in 2-propanol, acetone, toluene and *n*-octanol. The composition of the reaction mixtures was followed by absorption spectroscopy and high performance liquid chromatography. Photoprotection was negligible in *n*-octanol. In the other three solvents, the photoprotection of chlorophyll a with all-*trans*-β-carotene increased with decreasing viscosity and up to saturation with increasing concentration of the carotene. However, both pigments were degraded with similar kinetics. Product analysis showed that the photoprotection was limited by oxidation of all-*trans*-β-carotene to a product which itself acted as a oxidant to chlorophyll a. Photoprotection by 9-*cis*-β-carotene was in acetone 50% more efficient than that by the all-*trans*-isomer, but became limited during irradiation due to isomerization of the 9-*cis* to the all-*trans* isomer. The data show that under appropriate conditions 9-*cis*-β-carotene can be a better photoprotectant than the all-*trans* isomer.

Keywords: Carotenes; Chlorophyll; Photosensitization; Photoprotection; Photodegradation; Photosynthesis

1. Introduction

Carotenes are ubiquitous in photosynthetic organisms, where they are involved in multiple functions. They can act as light-harvesting molecules for photosynthesis in the blue and green spectral region, they can protect the photosynthetic apparatus by screening the light, by quenching chlorophyll triplets, singlet oxygen or radicals, and they have structural functions [1–10]. They are also discussed as receptor chromophores in photomorphogenesis [11,12]. Depending on the pigment and the native environment, the relative importance of these functions varies. The photoprotective capacity of β-carotenes is also used in photomedical applications [9].

An understanding of these different functions on the molecular level has increased considerably recently owing to advances in time-resolved methods, facilitating the investigation of ultrafast kinetics of the excited states, and to improved analytical techniques, facilitating the investigation of pigments in situ [13–20]. Radiationless relaxation of the optically allowed ^{2}S to the forbidden ^{1}S state, to the lowest triplet state, and to the ground state exhibits characteristic differences among the various carotenoids. The relative energies of the ^{1}S state with respect to associated chlorophylls [21] and of the ^{1}T state with respect to $^1O_2$ are of particular importance. Because of the number of double bonds, geometric isomerization is an important photoreaction. The different locations of 15,15′-*cis*-carotenoids and all-*trans*-carotenoids in purple bacterial reaction centres and antennas respectively [14,22] indicate, for example, functional differences among geometric isomers. A common triplet has been found for all-*trans*-β-carotene (*trans*-car) and the 13- and 15-*cis* isomers, whereas 9-*cis*-β-carotene (9-*cis*-car) exhibits a different triplet state [23].

The halotolerant green alga *Dunaliella bardawil*, which is capable of growing under high light intensities, accumulates unusually large amounts of β-carotene. While its major fraction is always the all-*trans* isomer, the synthesis of the (generally very rare) 9-*cis* isomer is selectively enhanced by high irradiances and may reach 40% of the total amount [24]. The biological significance of this conspicuous increase is still unclear, but one possibility could be a better photoprotection of the photosynthetic apparatus by the 9-*cis* than the all-*trans* isomer. Several studies have addressed this question with conflicting results [25–29]. To investigate this possibility further, we have studied the photodegradation of chlorophyll

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a (Chl a) in organic solution in the presence of either 9-*cis*-car or *trans*-car. Our data show that under appropriate conditions the *cis* isomer is a better photoprotectant than the *trans* compound.

2. Materials and methods

Chl a was purified [30] from spray-dried *Spirulina geitleri* (SOSA Texcoco, Mexico). *trans*-Car was purchased from Sigma. 9-*cis*-car was kindly donated by A. Ben-Amotz. All solvents were of high performance liquid chromatography (HPLC) or analytical grade and were used without further purification.

Absorption spectra were monitored with a diode-array spectrophotometer (Hewlett–Packard) and fluorescence spectra with a spectrofluorimeter (model F-2000, Hitachi). For absorbance measurements Chl a was used at concentrations of no more than 10 μM; for fluorescence measurements concentrations were no more than 0.1 μM.

For irradiation, a standard slide projector (150 W) with a cold-light reflecting mirror equipped with a 630 nm low-pass filter (Schott), was used. It provided a photon flux density of 4.8×10^{16} cm^{-2} s^{-1} in the wavelength range 640–670 nm, which was determined with a Li-Cor quantum radiometer, model Li 189. Generally, solutions were irradiated for 30 min. Absorption spectra were recorded every minute during the first 10 min of irradiation, and then every 5 min during the remaining 20 min. Dark controls were in all cases performed under identical conditions. Changes in Chl a concentration were taken from the absorbance at 660 nm. Those of the carotenoids were taken from the absorbance at 428 nm, corrected for the absorption of Chl a at this wavelength in the same solvent system. The kinetics of pigment degradation were analysed using the PHAKMAT fitting program (Jandel Scientific).

HPLC with a diode array detector (DAD) (Hewlett-Packard, model 8452A) was performed as described previously [31], using a silica gel column cooled to 7 °C [32]. The eluent was acetonitrile–methanol–tetrahydrofuran (42:50:8, by volume); the flow rate was 1.5 ml min^{-1}.

Fast atom bombardment (FAB) mass spectra were determined by W. Schäfer (MPI for Biochemistry, Martinsried) in an *m*-nitrobenzylalcohol matrix (liquid secondary ion mass spectrometry (SIMS) mode, Cs gun, 20 kV, MAT, model 900).

3. Results

3.1. Photoprotection of Chl a by trans- and 9-cis-car in different solvents

Irradiation with red light ($\lambda > 630$ nm, 4.8×10^{16} photons cm^{-2} s^{-1} at 640–670 nm) of air-saturated solutions of Chl a in acetone, toluene, 2-propanol and *n*-octanol caused Chl a bleaching (Fig. 1, Table 1). In the concentration range 4–15 μM Chl a during the first 30 min of irradiation the extent of bleaching was concentration independent (not shown); all subsequent experiments were therefore performed at a Chl a concentration of 5 μM, if not stated otherwise. The rates of degradation for Chl a were similar in acetone, toluene and *n*-

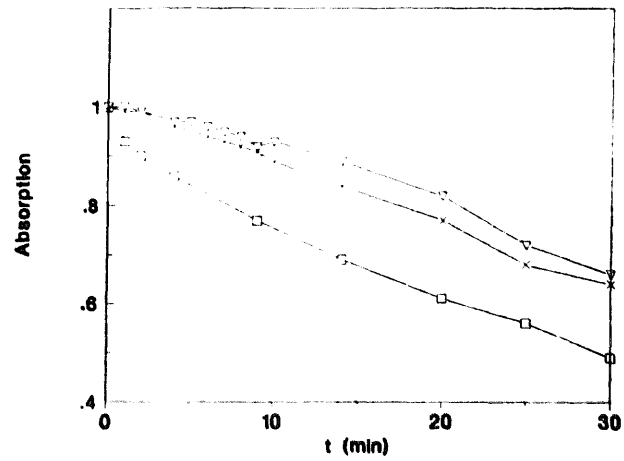

Fig. 1. Degradation of Chl a (▽, measured as absorption at 660 nm) and of *trans*-car (×, calculated from the absorption at 428 nm, see section 2) during irradiation of a pigment mixture (10 μM and 40 μM respectively) with red light ($\lambda > 630$ nm) in acetone. Degradation of Chl a (□, measured as absorption at 660 nm) irradiated in the absence of β-carotene under otherwise identical conditions.

Table 1
Photodegradation of Chl a in different solvents in the presence of increasing amounts of *trans*-car

Solvent	$c_{trans\ car}$ (μM)	d_{Chl} (%)	$k_{Chl} \times 10^4$ (s^{-1})	$k_{car} \times 10^4$ (s^{-1})
Acetone	0	60 ± 5	4.7 ± 0.3	–
	2.7	52 ± 5	3.8 ± 0.2	–
	3.9	49 ± 4	3.6 ± 0.3	4.0 ± 0.5
	13	44 ± 4	2.9 ± 0.2	4.2 ± 0.4
	26	30 ± 3	2.1 ± 0.2	2.2 ± 0.2
	39	36 ± 3	2.5 ± 0.1	2.2 ± 0.3
	52	38 ± 4		
	100	25 ± 2		
Toluene	0	67 ± 5	7.2 ± 0.4	–
	2.5	62 ± 5	5.6 ± 0.1	4.9 ± 0.3
	4	58 ± 5	4.0 ± 0.3	4.4 ± 0.3
	5	55 ± 4	4.5 ± 0.4	3.0 ± 0.3
	10	53 ± 4	3.9 ± 0.4	1.2 ± 0.2
	50	40 ± 4	2.9 ± 0.3	–
	100	38 ± 3	3.4 ± 0.3	–
2-propanol	0	38 ± 3	2.8 ± 0.3	–
	10	32 ± 3	2.1 ± 0.2	1.5 ± 0.3
	50	26 ± 3	1.7 ± 0.2	–
n-octanol	0	66 ± 6	6.5 ± 0.4	–
	5	66 ± 6	6.8 ± 0.3	0.7 ± 0.1
	10	68 ± 6	6.4 ± 0.3	0.6 ± 0.1
	50	64 ± 5	5.8 ± 0.4	–

Effective rate constants k_{Chl}, k_{car} and d_{Chl} for degradation rates of Chl a and *trans*-car and integrated destruction of Chl a respectively during 30 min irradiation are given. Chl a concentration was 5 μM throughout.

octanol, but considerably smaller in 2-propanol (60%, compared with the other solvents).

In the presence of *trans*-car, Chl a degradation was considerably reduced. The protection of Chl a by this carotenoid turned also out to be solvent dependent: the effect was best in acetone, lower in toluene and in 2-propanol, and no protection was found in *n*-octanol (Table 1). The protection correlates inversely with the viscosities of the solvents, which range in the order acetone (0.34 cP), toluene (0.6 cP), 2-propanol (2.9 cP) and *n*-octanol (10.6 cP) (all at 15 °C) [33]. The degradation kinetics of Chl a in the absence and presence of *trans*-car can be fitted by single exponentials. A summary of the rate constants is given in Table 1, and an example of the protection of Chl a in acetone is shown in Fig. 1.

The protection of Chl a depended on the irradiation time and the concentration of the *trans*-car. Fig. 2 shows this effect for acetone solutions. At short irradiation times, the protection was maximum at $c_{car} \geq 10$ μM and remained at this level at higher concentrations. Such a saturation effect was also seen at longer irradiation times, but saturation was then reached at progressively higher carotenoid concentrations. Qualitatively similar results were obtained with toluene and propanol as solvents (see Table 1). This effect can only be explained by the degradation of *trans*-car. Such a simultaneous photodegradation of *trans*-car became obvious from the results of two independent experiments with different methods. A spectrophotometric pigment analysis was performed in the 400–540 nm region after the subtraction of the Chl a absorbance (see section 2). It showed that in the "protective solvents" (toluene, 2-propanol, acetone), *trans*-car was destroyed at rates which are similar to those of the degradation of Chl a (Table 1; see also section 3.4). These results were further substantiated by HPLC analysis (see section 3.2).

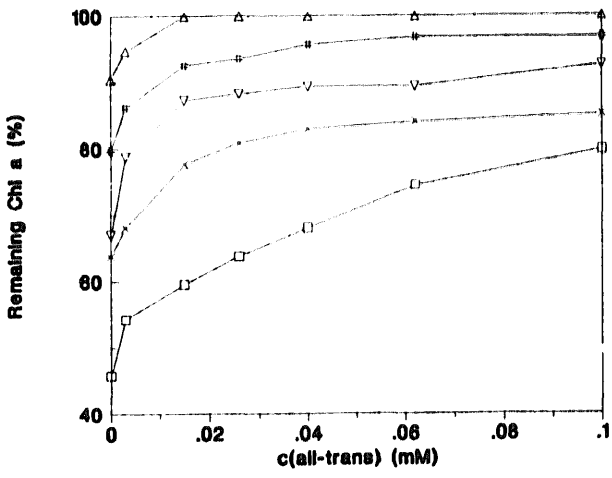

Fig. 2. Remaining Chl a (percentage of the initial concentration of 10 μM, in acetone) after irradiation (λ > 630 nm) in the presence of different concentrations of *trans*-car. Irradiation times were 5 (△), 10 (#), 15 (▽), 20 (×) and 30 (□) min.

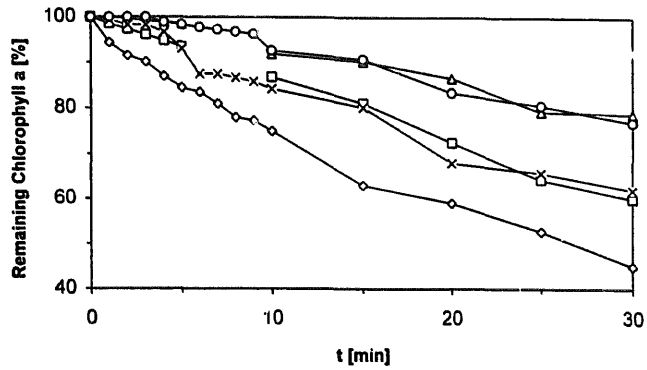

Fig. 3. Photodegradation of Chl a (5 μM in acetone) as determined spectrophotometrically at 660 nm: no additions (◇); in the presence of *trans*-car at $c = 13$ μM (□) and $c = 51$ μM (×); in the presence of 9-*cis*-car at $c = 13$ μM (△) and $c = 51$ μM (○).

Photoprotection of Chl a by the geometric isomer, 9-*cis*-car, was qualitatively similar to that of *trans*-car. Quantitatively, however, it was much more dependent on the solvent. When *trans*-car was replaced by 9-*cis*-car in acetone solution, the protection of Chl a was increased by 50% (Fig. 3). It was similar to that of *trans*-car in toluene and was not detectable (as for *trans*-car) in *n*-octanol. As a sensitized photoisomerization of 9-*cis*-car to *trans*-car is most likely, an equilibrium between the two isomers may be set up during irradiation [34]. It is thus proposed that the different solvent effects are due to different isomerization kinetics. This hypothesis was tested by following the reaction by HPLC, because it is difficult to follow this isomerization spectrophotometrically in the presence of Chl a and the degradation products of both pigments.

3.2. High performance liquid chromatography analysis of irradiated solutions of Chl a and trans-car

The irradiations were done as before, and aliquots were withdrawn at 5 min intervals over a total irradiation time of 30 min. Typical chromatograms of solutions of Chl a and *trans*-car, before (lower traces) and after (upper traces) 25 min irradiation with red light, are shown in Fig. 4. This depicts the absorbances at four characteristics wavelengths, which were taken from complete absorption spectra (350–820 nm) recorded at 3 s intervals. The main peaks (a and d) of the non-irradiated solution belong to Chl a ($t_r = 675$ s) and *trans*-car ($t_r = 1570$ s). The small peak (b) at $t_r = 745$ s is assigned to chlorophyll a′ (Chl a′) on the basis of its retention time [32] and its identical absorption spectrum to Chl a (trace A in Fig. 5, $\lambda_{max} = 662$ and 432 nm). During irradiation, the amounts of Chl a and *trans*-car decreased simultaneously. An increase is observed for the peak at $t_r = 745$ s, in particular in the 420 nm trace. Epimerization of Chl a to Chl a′ is known under a variety of conditions. However, another pigment with the same retention time must be formed, because the increase in visible mainly in the trace at 420 nm (Fig. 4). Chl a absorbs at both 420 nm and 662 nm, but no corresponding increase is seen at 662 nm, and the ratio of the blue and red maxima

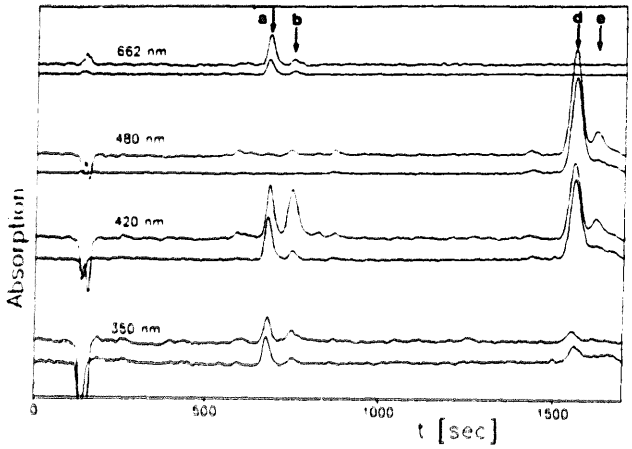

Fig. 4. HPLC–DAD analysis of the pigment composition of an acetone solution containing originally chlorophyll a (8 μM) and *trans*-car (18 μM) before (lower trace in each pair) and after (upper trace in each pair) irradiation for 25 min with red light (λ > 630 nm). Full spectra were recorded every 3 s, from which traces are shown at 350, 420, 480 and 662 nm. Peak labels: a, 675 s; b, 745 s; d, 1570; e, 1640 s. For clarity, the ordinates of the upper traces were multiplied by a factor of 1.04 (350 nm), 1.34 (420 nm), 1.63 (480 nm) and 2.09 (662 nm) in relation to the corresponding lower traces.

in the absorption spectrum is accordingly significantly increased (trace b in Fig. 5). An absorption spectrum of this newly formed pigment is shown in Fig. 5. It was obtained by subtraction of a Chl a spectrum from the spectrum recorded at $t_r = 745$ in a sample irradiated for 30 min, scaled such that the 662 nm peaks cancel. It is a carotenoid-type spectrum with maximum absorption at about 422 nm (trace d in Fig. 5). Compared with the *trans*-car present in the original solution, this maximum is blue shifted by nearly 40 nm. A similar blue-shifted absorption has been reported for aurochrome, a rearranged epoxide of β-carotene containing dihydrofuran rings [35]. Mass spectrometry of this product revealed a molecular ion at $m/z = 572$, proving that indeed this compound contains 2 atoms more of oxygen than *trans*-car. This is evidence for a Chl-a-sensitized photooxidation of *trans*-car. While the structural elucidation of the product was beyond the scope of this investigation, the results prove that *trans*-car is degraded in parallel to Chl a, and an oxygenation product is formed.

Another broad, small peak at a retention time of 1630 s in the HPLC appeared during the irradiation (e in Fig. 4). This peak contains 9-*cis*-car as identified by co-injection of an authentic sample, and by its spectrum (Fig. 5, trace c, $\lambda_{max} = 448$ nm). From peak integration of the traces at the maximum absorption of the broad peak, the *cis*-to-*trans* isomer ratio was determined as 0.28 in acetone and 0.25 in toluene.

Irradiated mixtures of Chl a and *trans*-car in *n*-octanol exhibited qualitatively the same composition as in the other solvents. Quantitatively, however, the ratio between the oxidation product ($t_r = 745$ s) and non-reacted *trans*-car after 30 min of irradiation was smaller than in acetone and toluene.

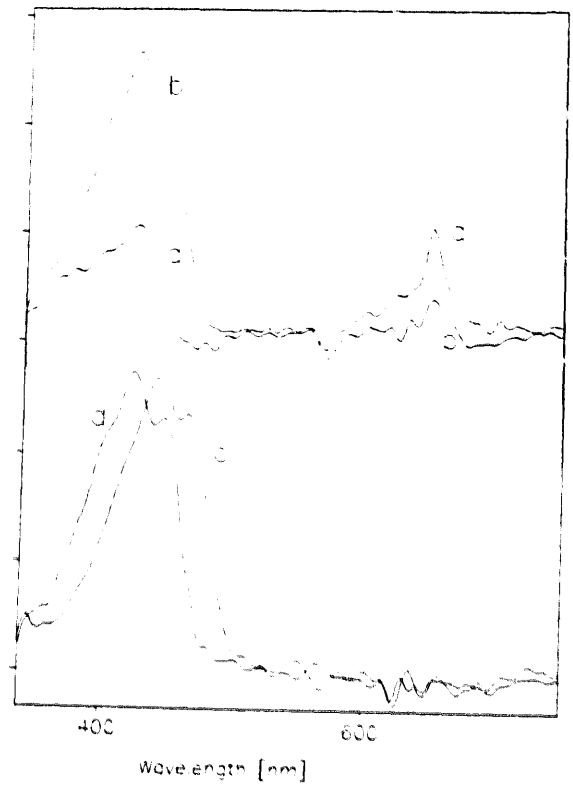

Fig. 5. Full spectrum of the 745 s HPLC peak shown in Fig. 3 before (spectrum a) and after (spectrum b) irradiation. Spectrum of 9-*cis*-car obtained from the 1640 s peak (spectrum c). Spectrum of the 745 s peak after subtraction of a Chl a spectrum obtained from the 675 s peak, normalized such that the 660 nm absorptions cancel (spectrum d).

3.3. Pigment analysis of irradiated solutions of Chl a and 9-cis-car

In order to evaluate the protection of Chl a by 9-*cis*-car, similar irradiation experiments were carried out in the presence of this isomer. These studies focused on acetone and toluene as solvents. Mixtures of Chl a and 9-*cis*-car were irradiated with red light for different times under the same conditions as used for the experiments with *trans*-car, and the pigments analysed as before by HPLC. The original β-carotene sample was contaminated with about 25% *trans*-car and by small amounts of a series of at least three other pigments ($t_r = 1380$–1500 s). α-carotene has been reported as a contaminant of β-carotene from *Dunaliella* by Ben-Amotz et al. [36].

On irradiation, the amount of 9-*cis*-car decreased rapidly with simultaneous formation of *trans*-car as the main product (Figs. 6 and 7). After approximately 20 min the ratio between 9-*cis*-car and *trans*-car reached a value of about 0.25; cf. the photoequilibrium value given above. Such a Chl-a-sensitized photoisomerization is well known [34]. The *cis*-to-*trans* conversion was dependent on the concentration of 9-*cis*-car; it decreased with increasing concentration. As in the experiments with *trans*-car, the peak at $t_r = 745$ s increased when detected at 420 nm (Fig. 7). According to its mobility and

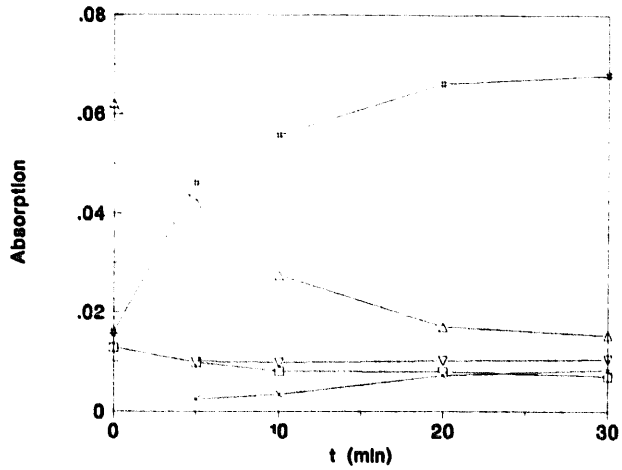

Fig. 6. Pigment composition of a solution of Chl a (5 μM initial concentration) and 9-*cis*-car (30 μM initial concentration) in toluene during irradiation with red light (λ > 630 nm). HPLC–DAD analyses of the peaks at 1640 s (△, 9-*cis*-car, 448 nm), 1570 s (#, *trans*-car, 454 nm), 675 s (□, Chl a, 662 nm), 745 s (×, oxidation product PO, 426 nm) and 1440 nm (▽, carotenoid impurity, see text, 448 nm). Plotted are the absorptions in the peak maxima at the given wavelengths.

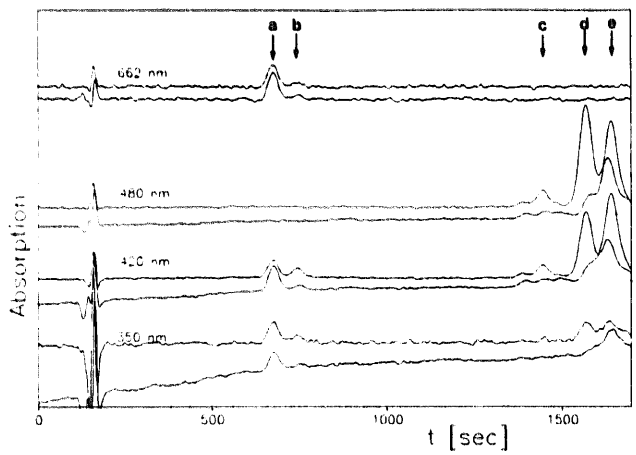

Fig. 7. HPLC–DAD analysis of the pigment composition of an acetone solution containing originally Chl a (8 μM) and 9-*cis*-car (30 μM) before (lower trace in each pair) and after (upper trace in each pair) irradiation for 10 min with red light (λ > 630 nm). Full spectra were recorded every 3 s, from which traces are shown at 350, 420, 480 and 662 nm. Peak labels: a, 675 s; b, 745 s; c, 1440 s; d, 1570 s; e, 1640 s. For clarity, the ordinates of the upper traces were multiplied by a factor of 1.38 (350 nm), 0.81 (420 nm), 2.81 (480 nm) and 0.85 (662 nm) in relation to the corresponding lower traces.

absorption spectrum, it contains the same oxidation product as found in irradiated solutions containing Chl a and *trans*-car. It is formed, however, with a delay relative to the appearance of *trans*-car, and after 30 min its yield was about 10% (Fig. 6). One of the three impurities at $t_r = 1380–1500$ s is relatively stable ($t = 1440$ s), while the others are degraded (Fig. 7).

Appropriate controls were done for all irradiations. In the dark, the composition of the 9-*cis*-car sample was stable under the reaction conditions. Surprisingly, a slow isomerization could be observed after irradiation with red light even in the absence of Chl a, although the low-pass filters used had a transmission of less than 0.1% below 600 nm. Carotenoid cation radicals have absorptions in the 900–1000 nm range, but these are not expected to be present in our solutions containing only 9-*cis*-car and solvent [3]. The forbidden $S_0 \rightarrow S_1$ transition of carotenoids is also expected in this region. Its extinction coefficient in *trans*-neurosporene has been estimated to be only 60 $cm^{-1} M^{-1}$ [17], which is far too low to explain the non-sensitized isomerization. There are to our knowledge no corresponding data available at present for 9-*cis*-car. Extinction coefficients above 2000 $cm^{-1} M^{-1}$ should be clearly seen in the absorption spectrum, which was, however, free of bands in the region above 600 nm. Yet another origin could be thermal isomerization, but this process is slow for 9-*cis*-car at the temperatures used [37]. We are therefore at present unable to rationalize this reaction.

3.4. Decay kinetics of Chl a, trans-car and 9-cis-car during the irradiation

The decrease in the Chl a absorption ($\lambda_{max} = 662$ nm) during the irradiation could in all experiments be fitted well monoexponentially. In acetone, toluene and *n*-octanol, $k_{Chl\ a}$ was $(5–7) \times 10^{-4}\ s^{-1}$. Its value was significantly smaller in 2-propanol ($(1.7–2.8) \times 10^{-4}\ s^{-1}$). In the presence of *trans*-car, $k_{Chl\ a}$ decreased in acetone, toluene and 2-propanol. Interestingly, the degradation rates of Chl a and *trans*-car were roughly pairwise similar in these solvents under all conditions (Table 1). In *n*-octanol, there was no significant decrease in $k_{Chl\ a}$ in the presence of *trans*-car, and no correlation was found between the rate constants for destruction of Chl a and *trans*-car (Table 1).

The same kinetic analysis was extended to the HPLC data (Table 2). The degradation of Chl a and *trans*-car could again

Table 2
Photoreaction of solutions of Chl a and *trans*-car in toluene and acetone

C_{car} (μM)	Solvent	$k_{Chl\ a} \times 10^4$ (s^{-1})	$k_{trans\text{-}car} \times 10^4$ (s^{-1})	$k_n \times 10^4$ (s^{-1})	$k_{9\text{-}cis\text{-}car} \times 10^4$ (s^{-1})
0	Toluene	6.6 ± 0.6	–	–	–
8		5.9 ± 0.6	4.7 ± 0.5	−5.1 ± 0.6	–
15		5.0 ± 0.6	4.9 ± 0.6	−4.7 ± 0.6	–
110		5.9 ± 0.6	–	−6.9 ± 0.7	−5.5 ± 0.7
18	Acetone	2.9 ± 0.3	3.2 ± 0.4	−2.9 ± 0.4	−3.2 ± 0.6
27		2.8 ± 0.3	–	−2.2 ± 0.4	–

be fitted monoexponentially, and the decay rate constants were in good agreement with those determined spectrophotometrically (Tables 1 and 2). There was only one exception to this kinetic behaviour: in acetone in the presence of 9-*cis*-car, the degradation of Chl a exhibited an induction period; there is hardly any degradation during the first minutes (Fig. 3). This corroborates the spectroscopic analysis, revealing a better photoprotection of Chl a in the presence of 9-*cis*-car, as long as the photoisomerization is slow and the 9-*cis*-car:*trans*-car ratio is far from equilibrium. There was no difference in the kinetics of photodegradation between 9-*cis*-car and *trans*-car in toluene and no photodegradation in *n*-octanol.

3.5. Quenching of Chl a fluorescence by trans-car

Addition of *trans*-car to a Chl a solution in acetone, toluene and 2-propanol resulted in (i) a decrease in the fluorescence intensity and (ii) in a small (about 1 nm) but distinct red shift of the Chl a fluorescence maximum (not shown). No fluorescence quenching was found in *n*-octanol. For the first three solvents the quenching of fluorescence in the presence of increasing amounts of *trans*-car was analysed by using the Stern–Volmer equation $I_0/I = 1 + \kappa c_q$, where I_0 and I are the intensities of fluorescence in the absence and presence respectively of quencher, κ is the quenching constant and c_q is the concentration of the quencher, e.g. *trans*-car. Our data can be fitted satisfactorily in all three solvents with $\kappa \approx 200\ M^{-1}$.

Such a high value of the quenching constant cannot be explained by dynamic quenching. It is typical for static quenching where a fraction of the fluorophores is complexed, in the ground state, to the quencher [38]. Literature data on the ability of *trans*-car ($c \approx 10\ \mu M$) to quench the fluorescence of chlorophylls are controversial. Quenching was found [39] for solutions containing 50 μM of chlorophyll b in 75% ethanol–water and with Chl a in nematic phases [40], indicative of complex formation (see also Ref. [41]). Beddard et al. [42] found fluorescence quenching of Chl a (4 μM concentrations) in benzene, but only for rather high *trans*-car concentrations (millimolar), and ascribed it to dynamic quenching induced by electron transfer. The conflicting results may be due to Chl a aggregation [40,41,43].

4. Discussion

Chl a is a well-known photosensitizer producing singlet oxygen ($^1O_2$) via a spin-allowed energy transfer from chlorophyll triplets (^{3}Chl) [8,17,44]. $^1O_2$ can then be quenched physically or react chemically with the components of the solution. Quantitatively, *trans*-car ($k_q \approx 1.4 \times 10^{10}\ M^{-1}\ s^{-1}$ [14]) is a much better quencher of $^1O_2$ than Chl a ($k_q = 2 \times 10^8\ M^{-1}\ s^{-1}$ in benzene [45]), which explains its protective role. The corresponding rate constant for 9-*cis*-car is unknown. The data presented here show that in acetone solution it is a better protectant than the all-*trans* isomer. However, this protection is limited by the rapid isomerization to *trans*-car which proceeds via the same triplet state involved in $^1O_2$ quenching.

In toluene, there is no difference in protection among the two carotene isomers, and the induction period for the oxygenated carotenoid ($t_r = 745$ s) is lacking. One possibility to explain the different behaviour in acetone and toluene is that in the latter the isomerization is so rapid that although *cis*-car is principally again the better protectant, it never becomes effective. However, there is even no difference in protection at high starting concentrations (10^{-4} M and above), where 9-*cis*-car was still present in considerable amounts after 30 min. The equal protective power of both isomers must then be due to similar rate constants of $^1O_2$ quenching among the isomers, illustrating how critical the environment is in modulating the relative protective power.

In the discussed case protection can occur mainly via energy transfer directly from triplet Chl a, or from $^1O_2$ to carotene. The first route is rate controlled, an the second is controlled by diffusion of the small oxygen molecule. Our finding of an inverse correlation of the protection with the solvent viscosity indicates that the second route is favoured in this particular case.

However, this mechanism does not explain the saturation behaviour of Chl a destruction for varying *trans*-car concentrations shown in Fig. 2. A simple calculation shows that, for a reaction constant of $2 \times 10^8\ M^{-1}\ s^{-1}$ [45], the amount of Chl a, which will be oxidized by singlet oxygen and therefore cannot be protected, does not exceed 10%. By contrast, even for the highest *trans*-car concentration used (100 μM) more than 30% of the Chl a was oxidized during the experiment. This low protection can, however, be rationalized if another oxidizing agent is formed in the system. Our results suggest that such an agent is indeed formed: it is a chemical product of *trans*-car and singlet oxygen. This is concluded from the following data. (1) There is a kinetic correlation between Chl a and *trans*-car degradation rates. (2) An oxygenation product of *trans*-car ($t_r = 745$ s) has been identified, which contains two oxygen atoms. It has been suggested that, during the oxidation of *trans*-car, peroxyl-β-carotene adducts can be formed [46,47]. We propose that such peroxide(s) are the reactive intermediate(s) which oxidize Chl a or alternatively rearrange to the doubly oxygenated carotene. (3) The formation of this stable product, and hence also of its hypothetical precursor, is expected to be limited by the concentration of singlet oxygen and indirectly by that of Chl a. This is in agreement with the observation that the amount of the product eluting at 745 s is almost independent of the *trans*-car concentration. (4) The formation of the 745 s product is delayed when solutions of 9-*cis*-car are irradiated in the presence of Chl a. This delay relates kinetically to the isomerization of 9-*cis*-car to *trans*-car. We therefore propose that the 745 s product and hence also its hypothetical precursor are only formed from *trans*-car.

In addition, the 9-*cis*-car configuration might be more advantageous in dissipating the transferred triplet energy

from Chl a with subsequent isomerization into *trans*-car. It was shown that 9-*cis* isomers of carotenoids, in contrast to the 11-, 13- and 15-*cis* isomers, can generate their own triplet states different from those of the corresponding *trans*-car, which can efficiently ($\Phi = 0.15$) isomerize into *trans*-car [6]. Accordingly, this situation is only transitory owing to the very fast 9-*cis*-car → *trans*-car isomerization and subsequent formation of the oxygenation product at $t_r = 745$ s (Fig. 6). Additional comparison of triplet state properties of both isomers is required for a better understanding of this problem.

A shift in the fluorescence emission band of Chl a in the presence of *trans*-car and a high value of the quenching constant (typical for static quenching) in acetone, toluene and 2-propanol, can be an indication for the formation of a ground state complex between the molecules [46]. However, the concentration of such complexes is less than 2% of the Chl a concentration (see above), and they do not seem to play a major role in the protection process with *trans*-car. Obviously, such ground state complexes between Chl a and *cis*- or *trans*-car can provide another mechanism of differentiation among the two isomers.

5. Conclusions

(1) Photoprotection of Chl a by *trans*-car is limited by the side reaction of *trans*-car oxidation. The hypothetical peroxide formed by *trans*-car oxygenation can react itself as oxidant to Chl a.

(2) Photoprotection of Chl a by all-*trans*- and 9-*cis*-car depends on the solvent. It decreases with increasing viscosity. In acetone, it is better for the *cis* than for the *trans* isomer but limited by the rapid sensitized *cis*-to-*trans* isomerization. In toluene, we found no such difference.

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