# Chlorophyll Photobleaching in Pigment-Protein Complexes

Robert Carpentier, Roger M. Leblanc

Centre de recherche en photobiophysique, Université du Québec à Trois-Rivières,
Trois-Rivières, Québec, Canada G9A 5H7

and

Guy Bellemare

Département de biochimie, Faculté des sciences et de génie, Université Laval, Ste-Foy, Québec,
Canada G1K 7P4

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Pigment photobleaching was performed in thylakoid membranes of *Hordeum vulgare* (wild type, mutant Chlorina f2, Norfluranzon treated seedlings) and in pigment-protein complexes (CP-I and LHCP) isolated from *H. vulgare* and *Chlamydomonas reinhardtii.* Multiphasic kinetics were obtained in all of the above cases. Energy transfer towards pigments absorbing at longer wavelength is postulated as a general protection mechanism against photobleaching. This mechanism explains a substantial bleaching of carotenoids and a faster bleaching of chlorophyll aggregates, absorbing at long wavelength. These conclusions were valid for isolated complexes as well as for thylakoid membranes, although membranes were less sensitive to light.

## Introduction

In the thylakoid membranes, photosynthetic pigments are specifically associated with proteins to form pigment-protein complexes [1–3]. There is strong evidence that all of the pigment molecules are tightly bound to these complexes [4, 5]. Three types of pigment-protein complexes have been shown to exist: (i) the CP-I complex containing the reaction center $P_{700}$, Chl *a* and carotenoids, (ii) the CPa complex containing the reaction center $P_{680}$, Chl *a* and carotenoids and (iii) the LHCP complex containing Chl *a,* Chl *b* and carotenoids. CPa contains only a small proportion of the total pigments, which are mainly associated with CP-I and LHCP [2, 6, 7]. However, LHCP functions as an antenna mainly oriented towards the $P_{680}$ [2].

Following light absorption, the pigments are in part protected from photobleaching (photooxidation) by energy dissipation through the electron transport [8] and oxidized molecules are replaced by newly synthesized ones. Nonetheless, not all of the absorbed energy is used by the photosynthetic process and a photoprotection mechanism is necessary to prevent an abusive Chl bleaching. Pigment photobleaching has been studied in the internal membrane of the chloroplast [9–12] and in vesicles made with chloroplast lipids [13]. These experiments indicated that energy transfer towards longwavelength absorbing holochromes (Chl aggregates or carotenoids) is a potent protection mechanism against oxidation of pigments absorbing at shorter wavelengths. However, no data related to the photobleaching of isolated pigment-protein complexes has been published. Since these complexes represent the basic unit of pigment integration inside thylakoid membranes, we present extended studies of this reaction to further investigate the photoprotection mechanism. Photobleaching of isolated complexes and thylakoid membranes are compared in order to verify if the membrane organization alterates the pigment photosensitivity.

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*Abbreviations:* Chl, chlorophyll; Chls, chlorophylls; LDS, lithium dodecyl sulfate; CP-I, chlorophyll-protein complex I; CPa, chlorophyll-protein complex a; LHCP, light-harvesting chlorophyll *a*/*b* protein.

Reprint requests to Dr. R. Carpentier.

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## Materials and Methods

LDS was purchased from Gallard-Schlesinger, Carle Place, NY. Most of the chemicals were from Sigma, St. Louis, MI.

Culture of *Hordeum vulgare* and *Chlamydomonas reinhardtii* – Seedlings of wildtype barley and the mutant chlorina f2, lacking Chl *b*, were grown in a greenhouse. In the case of seedlings treated with Norflurazon (SAN-9789), the herbicide was added to tap water at a concentration of 28 mg $l^{-1}$ ($10^{-4}$ M) and the ambient light intensity was reduced by covering the seedlings with white towels. The green alga *C. reinhardtii* (strain 137c) was grown at 25 °C in sterile minimal Tris-phosphate medium, according to Sager and Granick [14]. Cells were collected at the end of the exponential growth phase.

Isolation of membranes and pigment-protein complexes – Membranes were isolated from *H. vulgare* leaves according to Machold *et al.* [2], or from *C. reinhardtii* cells according to Delepelaire and Chua [7], in the dark at 12 °C, and were stored at −40 °C. CP-I and LHCP complexes were isolated from thylakoid membranes by gradient polyacrylamide gel electrophoresis at 4 °C as described by Piccioni *et al.* [15] and electroeluted (7 mA, 4 h) in 0.05 M Tris – 0.38 M glycine buffer pH 8.3. This same buffer was used for storing the complexes [16]. Chl was determined in 80% acetone according to Arnon [17].

### *Photobleaching*

Photobleaching experiments were performed according to Dinant and Aghion [18]. The incident light intensity from a quartz-halogen lamp fitted with an infrared filter was 400 ± 50 W · $m^{-2}$. Chl *a* concentration was kept between 1–3 mg $l^{-1}$ in all experiments. The results were given as percent Chl *a* bleached per minute at the red absorption maximum. Absorption spectra were recorded with a Cary 17D spectrophotometer.

## Results

### *Photobleaching kinetics*

The photobleaching of photosynthetic pigments adsorbed on proteolipid particles [18] or inserted into vesicles made with either chloroplast lipids [13] or phosphatidylcholine [19] was characterized by a monophasic second order kinetic. However, this reaction followed at the maximum of Chl absorption in the red for CP-I and LHCP isolated from *H. vulgare* and *C. reinhardtii* presents a much more complicated figure (Fig. 1a, b). In CP-I, the Chl photobleaching starts after a lag time of 1 min and presents a marked decrease of velocity between 14 and 20 min of light exposure when 9 to 11% of the Chl is bleached. However, in the case of LHCP, there is no lag time before the initiation of bleaching (Fig. 1b). In the latter, the velocity of the reaction decreases between 9 and 13 min and, more remarkably, between 15 and 22 min after 17 to 18% and 22 to 23% of the Chl is bleached. The initial bleaching velocity is about three times higher for LHCP than for CP-I (Fig. 1a, b).

Complex kinetic behavior occurs not only in the case of pigment-protein complexes isolated from two different organisms, but also when thylakoid membranes are photobleached (Fig. 1). Three types of membranes isolated from barley were exposed to light: (i) wild type, (ii) mutant chlorina f2 and (iii) wild type treated with Norflurazon (SAN-9789) (an herbicide which inhibits carotenoid biosynthesis [20, 21]). The bleaching kinetics of these membranes are reproducibly biphasic (Fig. 1c). The kinetics obtained from membranes isolated from Norflurazon-treated seedlings show a lag of 1 min before the beginning of bleaching and a decrease of velocity between 20 and 25 min of light exposure. The lag is about twice as long for the other membranes, and they exhibit a decrease of bleaching velocity between 13 and 15 min. Wild-type chloroplast membranes bleach slightly more rapidly than those isolated from the mutant chlorina f2. However, membranes from leaves treated with the herbicide are much more light sensitive, since the initial bleaching velocity is three times higher than for the other types of membranes (Fig. 1c).

From the initial bleaching velocities given in Fig. 1, we can classify the materials studied by order of increasing velocities: [membranes from wild type] < [membranes from Chlorina f2] < [CP-I] < [membranes from Norflurazon-treated seedlings] < [LHCP]. This same order would also be valid if we consider the percentage of bleached Chl after 30 min of light exposure.

### *Photobleaching spectra*

During the course of photobleaching experiments, the absorption maximum of Chl *a* in the red is gradu-

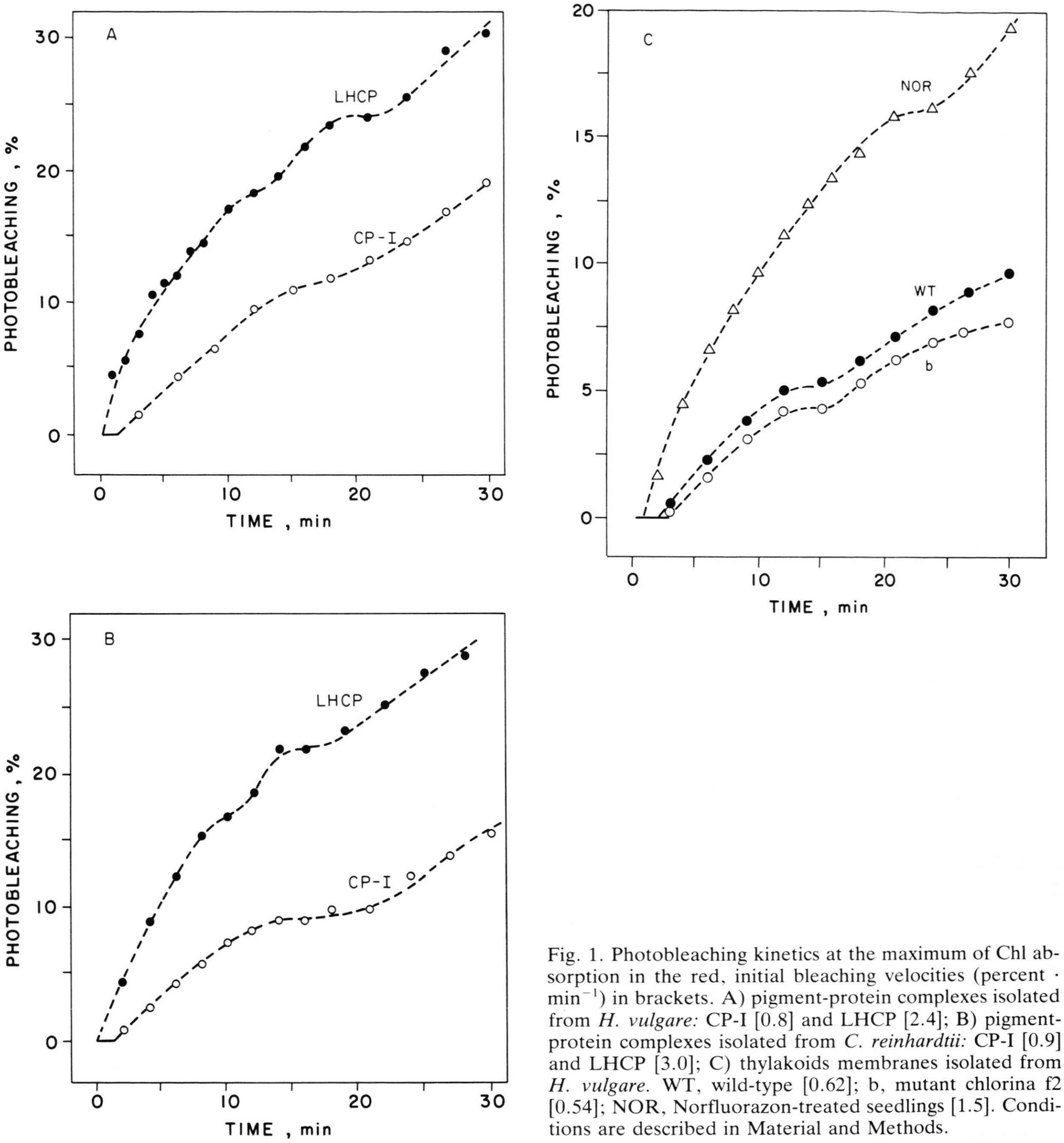

Fig. 1. Photobleaching kinetics at the maximum of Chl absorption in the red, initial bleaching velocities (percent · $\text{min}^{-1}$) in brackets. A) pigment-protein complexes isolated from *H. vulgare:* CP-I [0.8] and LHCP [2.4]; B) pigment-protein complexes isolated from *C. reinhardtii:* CP-I [0.9] and LHCP [3.0]; C) thylakoids membranes isolated from *H. vulgare.* WT, wild-type [0.62]; b, mutant chlorina f2 [0.54]; NOR, Norfluorazon-treated seedlings [1.5]. Conditions are described in Material and Methods.

ally shifted towards shorter wavelengths. The extent of this wavelength shift after 30 min of light exposure is variable (1–3 nm) but is always in the same direction (Figs. 2 and 3). The absorption maximum of Chl *a* in the difference spectra calculated from absorption spectra measured before and after 30 min of light exposure (Figs. 2 and 3) appears at a wavelength 1 to 6 nm longer than the same maximum in the initial absorption spectra (before bleaching). The above results clearly indicate that Chl aggregates absorbing at longer wavelengths are more bleached.

Carotenoid bleaching is also quite evident since the difference spectra in Figs. 2 and 3 present a strong absorption between 450 and 530 nm with an exception in the case of the membranes isolated from plants treated with Norflurazon and lacking carotenoids (see Fig. 3c). A maximum seen at 555 nm in Fig. 3c is probably due to cytochrome absorption. When present, Chl *b* is much less bleached than Chl *a*; this fact is most evident in the case of LHCP, where the shoulder due to Chl *b* absorption, appearing at 665 nm in the spectrum of LHCP, increases gradually during light exposure in comparison to the absorbance of Chl *a* (see Fig. 2b).

## Discussion

The material used in this study contains pigment holochromes absorbing at different wavelengths. The degree of Chl orientation with the plane of thylakoid membrane increases with the wavelength of the absorption maximum in the red [22]. This phenomenon probably coincides with the higher energy transfer efficiency towards Chl aggregates absorbing at longer wavelengths [23]. In previous photobleaching experiments, it has been shown that the presence of Chl *a* aggregates absorbing at 700 nm (Chl $a_{700}$) in vesicles made with chloroplast lipids greatly reduces the bleaching of the major form of Chl *a* (Chl $a_{669}$) [13]. This result has been explained in terms of a protection mechanism involving energy transfer from Chl $a_{699}$ to Chl $a_{700}$. The energy transfer process has also been proposed as an explanation for the Chl bleaching in the internal membranes of the chloroplast [9]. For example, in the thylakoid membranes, the Chl holochrome absorbing at 680 nm bleaches more rapidly than the one absorbing at 670 nm, and in photosynthetic algal cells, the holochrome absorbing at 693 nm is the most light sensitive. The results described in this report indicate that this process is involved at the level of the pigment-protein complexes, not only when they are inserted in the thylakoid membranes, but also when they are isolated by detergent fractionation. In fact, the absorption spectra (Figs. 2 and 3) show that the Chl aggregates absorbing at long wavelengths are more bleached.

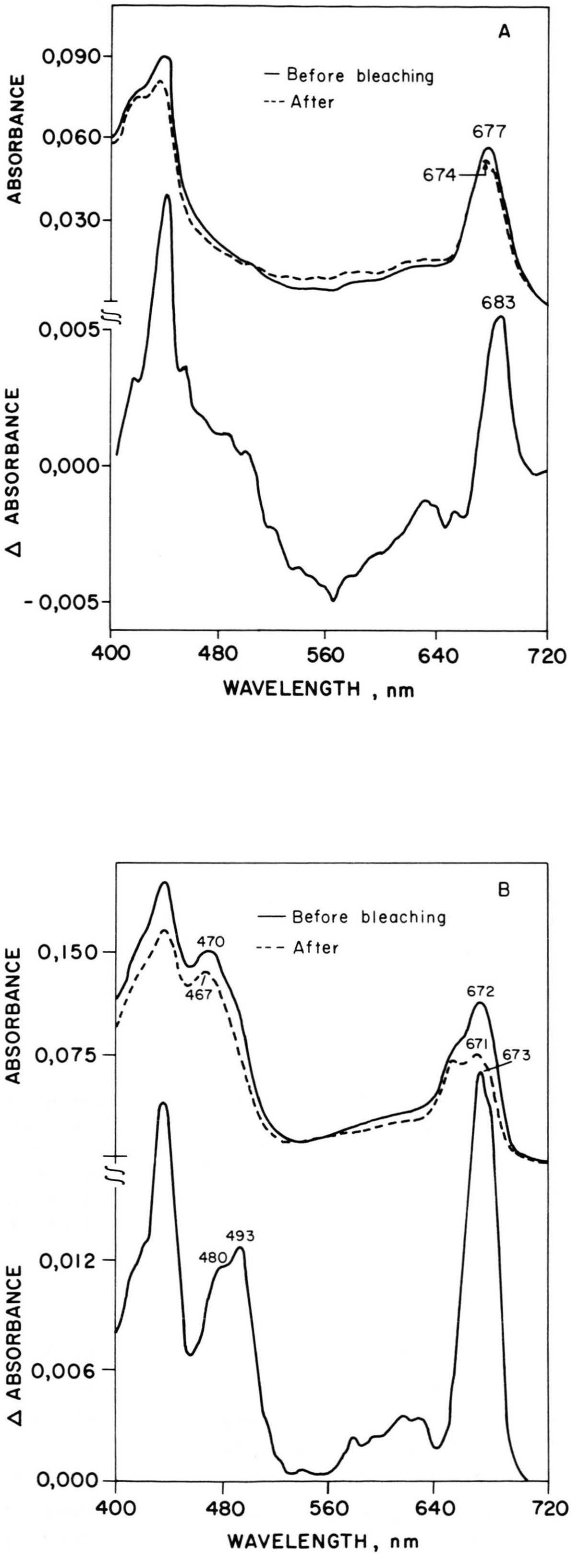

Fig. 2. Absorption spectra of pigment-protein complexes isolated from *H. vulgare:* A) CP-I; B) LHCP. Upper part, spectra recorded before and after 30 min of light exposure; lower part, the difference spectrum calculated between the spectra of the upper part after their normalization at 720 nm.

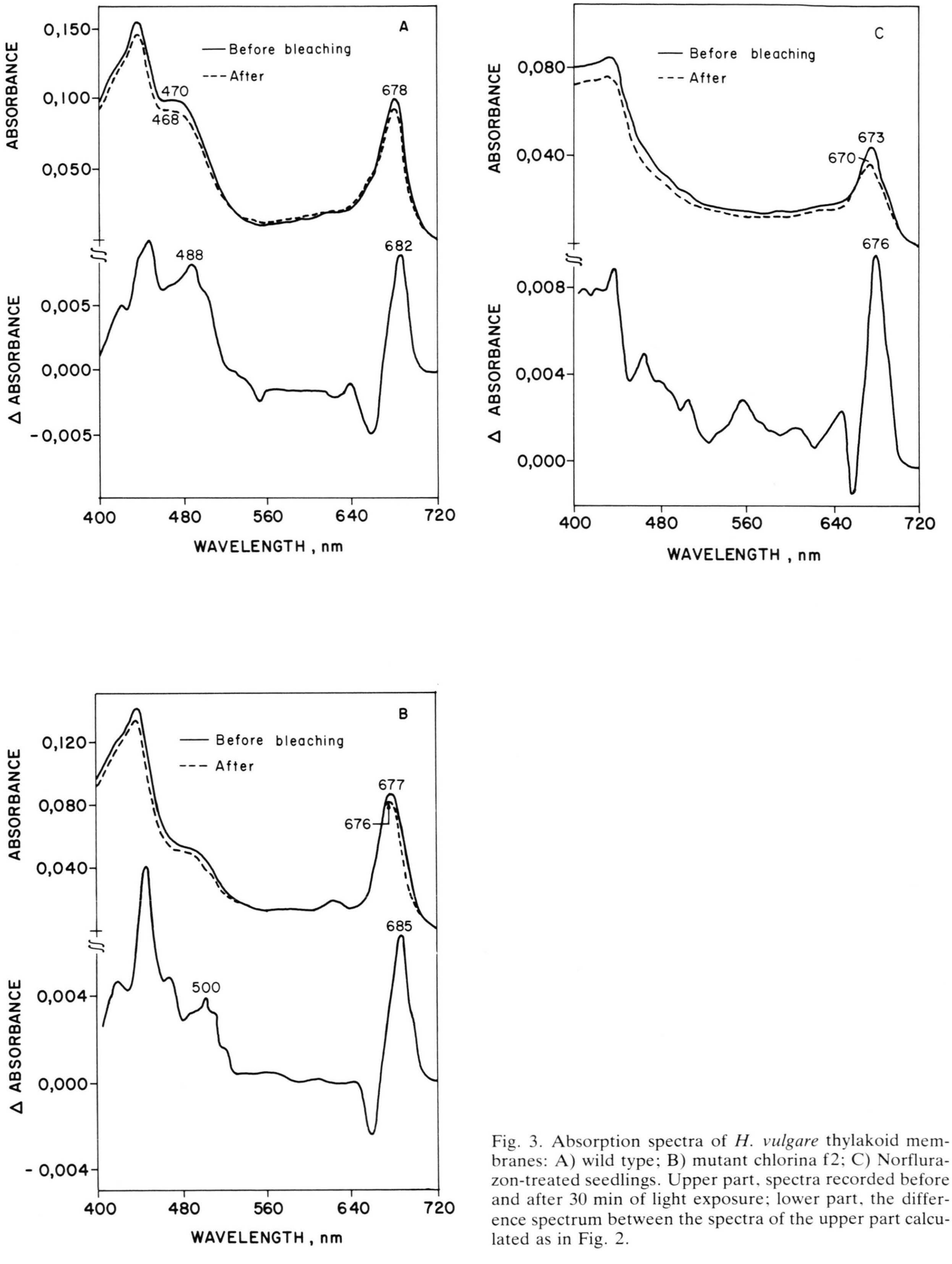

Fig. 3. Absorption spectra of *H. vulgare* thylakoid membranes: A) wild type; B) mutant chlorina f2; C) Norflurazon-treated seedlings. Upper part, spectra recorded before and after 30 min of light exposure; lower part, the difference spectrum between the spectra of the upper part calculated as in Fig. 2.

Energy transfers from Chl *b* and carotenoids to Chl *a* have been observed from fluorescence measurements in barley pigment-protein complexes isolated under conditions similar to those used in the present study [6, 24]. Energy transfers are also detected by photobleaching experiments. Indeed, a slower photobleaching rate of Chl *b*, due to energy transfer to Chl *a*, was reported for chloroplast membranes [11, 12]. We have observed this phenomenon at the level of LHCP complexes where a shoulder due to Chl *b*, located at 655 nm in the absorption spectra, increases after light exposure in comparison with the absorption maximum of Chl *a* (Fig. 2b). Furthermore, the ability of carotenoids to protect Chl *a* from light suggests that a large part of the carotenoid bleaching seen in Figs. 2 and 3 is due to triplet-triplet energy transfer from Chl *a* to carotenoids [25, 26]. The above discussion supports the idea of a protection mechanism where Chl *a* is protected by Chl aggregates absorbing at longer wavelengths and the latter by carotenoids, which receive the overflow of absorbed energy (see also ref. [27]).

The different bleaching rates of the thylakoid membranes (Fig. 1c) can be explained in terms of the pigment-protein complexes content. For example, membranes from plants treated with the herbicide bleach rapidly because they contain only a small quantity of carotenoids and Chl *a* absorbing at long wavelengths (Fig. 3c). The position of the maximum of Chl *a* absorption in these membranes (673 nm) indicates a low level of long-wavelength aggregated forms of photosynthetic pigments.

In isolated complexes, LHCP bleaches more rapidly than CP-I (Fig. 1a, b). This rapid bleaching should be explained by a low content in holochromes absorbing at long wavelengths in LHCP compared to CP-I and by a greater light absorption efficiency due to the presence of Chl *b*. Thornber and Highkin [28] have shown that membranes from the mutant chlorina f2 do not contain LHCP. Chl *a* is then associated with the two other types of complexes (CP-I and CPa), both of which contain a photochemical reaction center (CP-I contains $P_{700}$ and CPa contains $P_{680}$) [29–31]. Due to energy transfer between LHCP and CPa in the membranes isolated from the wild-type, they bleach only 10 to 15% faster than the ones of chlorina f2 (Fig. 1c). In general, slower bleaching rates for thylakoid membranes than for isolated pigment-complexes indicate a decrease in energy transfer yield within the complexes following isolation or, more likely, energy transfer between complexes in the membranes. The former explanation is not sustained by the high yield of energy transfer found in complexes isolated by electrophoresis [6, 24].

It is also relevant to note that the different complexes have a different content of carotenoids [32, 33]. Even if all carotenoid classes are all good singlet oxygen quenchers in solution [25], they do not have the same interaction with Chl *a*. For example, some are known to have coordinated interactions with Chl *a*, while others do not [34, 35]. This variation in the quantity and in the carotenoid classes should also affect the bleaching rates.

Finally, we observe multiphasic kinetics of photobleaching in isolated pigment-protein complexes and thylakoid membranes. It is unlikely that this situation could be explained by the formation of intermediates during Chl bleaching because monophasic kinetics were obtained with Chl inserted in chloroplast lipid vesicles [13] or adsorbed on proteolipid particles [18]. We rather suggest that these multiphasic kinetics represent the bleaching of different Chl populations which are sensitive to light at different levels, depending on their organization. For example, it has been suggested for PS-I [36] and LHCP [37] that Chls and carotenoids might be inserted within hydrophobic areas of the apoprotein core. It is possible that the apoprotein hydrophobic areas nearest to the pigment-protein complex edge could contain some lipid (lipid and detergent in the case of isolated complexes). In fact, it has been shown that up to 10–20% of thylakoid lipids remain attached to the pigment-protein complexes isolated by electrophoresis even if a large proportion of Chl molecules is released as free pigment [38, 39]. In these areas, the pigments would be diluted by lipids and have larger intermolecular distances, leading to a decreased yield of energy transfer and a faster bleaching of this Chl population.

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