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RELATIVE STABILITY OF CHLOROPHYLL COMPLEXES *IN VIVO*

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SUMMARY

The time course of aerobic photobleaching of various chlorophyll–protein complexes *in vivo* at high light intensities was studied with isolated *Aspidistra elatior* chloroplasts.

1. C_a680 bleaching starts with the onset of irradiation and, initially, proceeds linearly with time. Washing the chloroplasts causes a nearly constant increase of the bleaching rate throughout the experiment.

2. C_a670 does not appreciably, if at all, bleach initially; subsequently, bleaching proceeds linearly with time and at a slightly higher rate than that for C_a680. Washing makes C_a670 bleach concomitantly with the onset of illumination, and at a nearly constant rate.

3. Bleaching at 665 nm is likely to start only after a relatively long period of illumination. Washing shows no effects during this period. Once bleaching has started, washing causes its rate to increase.

4. No indication of the occurrence of "short-wave" chlorophyll *a* forms other than C_a670 and C_a665 was obtained.

5. C_b bleaching starts concomitantly with illumination at a low rate. The rate increases more or less exponentially with time. Washing enhances bleaching in two steps.

6. The importance of the results is discussed.

INTRODUCTION

The red absorption band of chlorophyll *a in vivo* shows a number of weak shoulders, in addition to the main maximum (*cf.* ref. 1), which are generally thought to correspond to the same number of chlorophyll–protein forms, each one absorbing at a slightly different wavelength. According to the approximate wavelengths of maximum absorption, these complexes are named C_a670, C_a680, C_a695 and C_a700. The latter complex may well be identical with KOK's[2] P700 and C_{700}. Experiments with Aspidistra chloroplasts and Anacystis cells provided evidence that some additional forms, namely C_a660, C_a664, C_a667 and C_a678 may occur[3,4]. Since the shoulders do not always show up, their occurrence was studied statistically. An attempt was

Abbreviations: C_a700, C_a695, C_a680, C_a670, C_a665, chlorophyll *a*–protein complexes *in vivo* with absorption maxima around 700, 695, 680, 670, and 665 nm, respectively; C_b, chlorophyll *b*–protein complex *in vivo*; DCIP, 2,6-dichlorophenolindophenol.

made to demonstrate the presence of these additional forms more clearly by mild acetone extraction[5], heat treatment[6], chromatography[7] and fluorescence[8]. In the two latter studies, spinach chloroplasts were used. With these chloroplasts (*cf.* ref. 7) a single chlorophyll type absorbing around 665 nm rather than two forms, C_a664 and C_a667, seemed likely to occur. BRIANTAIS[9] observed and partially isolated a C_a665 form with corn chloroplasts. The absorption shoulders due to the C_a660 and C_a678 forms were found[3,4] to be still weaker than that of C_a665. In none of the studies mentioned[5–8] was confirmation about their occurrence obtained. BRIANTAIS[9] did not observe these two forms in absorption spectra at liquid-nitrogen temperature. METZNER[10] reported the observation of 9 maxima and shoulders in the red chlorophyll *a* band of freeze-dried Chlorella cells. CEDERSTRAND, RABINOWITCH AND GOVINDJEE[11], who originally agreed upon the likelihood of the occurrence of more chlorophyll forms than the generally accepted ones, changed their opinion as their former results were obtained with a noisy instrument. BACON AND HOLDEN[12] demonstrated and discussed the fact that chlorophylls *a* and *b* are readily changed by chemical and physical treatments. Moreover, the composition of the photosynthetic pigment apparatus varies with age (*cf.* ref. 13).

Consequently, the number of chlorophyll *a* forms occurring *in vivo* is not yet definitely established, and further study is needed in this field. The present experiments may contribute to this purpose. As the various chlorophyll forms might differ in light stability, use was made of aerobic photobleaching of isolated Aspidistra chloroplasts. The changes in the red absorption band with time were recorded as difference spectra. In this way, the individual chlorophyll *a* forms might show up more clearly. Moreover, no structurally or chemically induced changes would disturb the results. There might be a risk that, concomitantly with bleaching, a shift of the absorption bands would occur. In such a case additional shoulders in the absorption spectrum are likely to arise. Such anomalies, however, were not observed in the present experiments.

MATERIAL AND METHODS

A freshly collected, full-grown *Aspidistra elatior* leaf, or a part thereof, was washed and rinsed with 0.02 M phosphate buffer (pH 7.4) and minced in a Braun multipress provided with a strip of filter paper along the wall of the perforated trough. The resulting juice was taken up in the same buffer and filtered through filter paper, yielding a "crude preparation". "Washed preparations" were obtained by centrifuging the crude preparation at 15000 × *g* for 15 min, and taking up the sediment in the buffer mentioned. All manipulations were carried out in as dim a white light as possible and in the cold.

The concentration of the preparations was adjusted by diluting with buffer until the absorbance at the red maximum amounted to about 0.7. For the preparative measurements as well as some preliminary experiments, a Beckman DK 2 recording spectrophotometer was used. The definitive spectra were recorded with a Cary Model 14R instrument, using slide wire 1480670 in the range 0–0.5.

For irradiation with white light a cuvette containing the suspension was placed in a refrigerated box in front of a 9-mm-thick perspex window. Outside this box, at a distance of 22 mm from the front wall of the cuvette, and 2 mm from the perspex

window to the front of the lens holder, a Cimator projector was placed and focused in such a way that at the front wall of the cuvette a light intensity of about 0.3 W/cm^2 was obtained.

PROCEDURE

The absorption spectrum of the preparation was obtained. Next difference spectra of irradiated *vs.* non-irradiated samples were recorded. For each measurement a fresh control sample was drawn from the bulk preparation which was kept in the dark under refrigeration.

The recorded difference spectra were multiplied by a factor: height of the maximum of the absorption spectrum (*a*) divided by that of the difference spectrum (*n*), thus yielding a "normalized" difference spectrum, and the sign of the latter spectrum was reversed. In this way, readily comparable spectra were obtained (*cf.* Fig. 1). In order to evaluate the bleaching of the various components, three cases are to be distinguished: (1) no overlap of the components; (2) overlap of known components; (3) overlap of unknown components.

Actually, Case 1 does not occur. However, chlorophyll *b* bleaches considerably

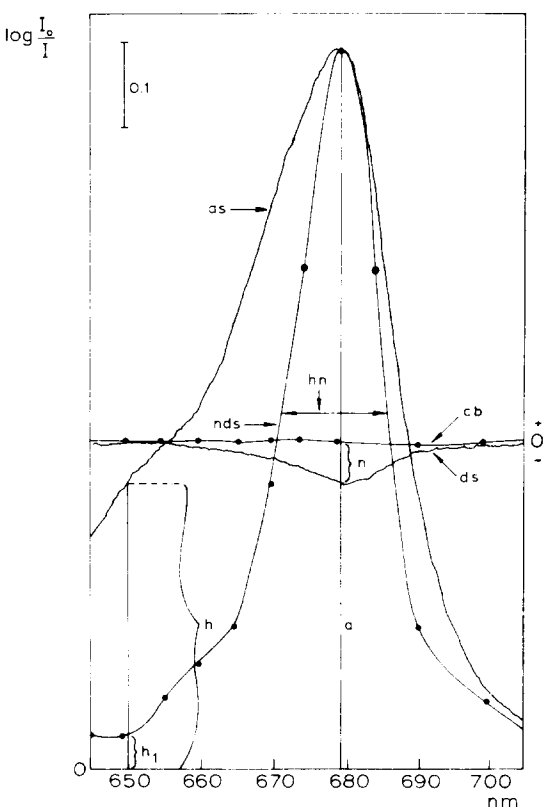

Fig. 1. Example of procedure. as: tracing of absorption spectrum; ds: tracing of difference spectrum 2.5 min irradiated *vs.* dark; cb; corrected base line for ds; nds: "normalized" difference spectrum; hn: half width. For further explanation of *a*, *n*, *h*, h_1, and the use of data, see text.

more slowly than the chlorophyll *a* forms do. Therefore an approximation of the C_b reaction could be obtained by disregarding the initially negligible overlap in the difference spectra. In such a way, an approximate percentage of C_b bleaching was established according to:

$$\frac{h_1 n}{ah} \cdot 100,$$

where h is the height of the absorption spectrum at 650 nm, and h_1 is the height of the "normalized" and reversed difference spectrum at this wavelength (*cf.* Fig. 1).

For Case 2 use was made of the half-width values of the absorption and difference bands, and the components considered are the major forms C_a680 and C_a670. To enable evaluation, the following assumptions are made.

(a) The concentration ratio C_a680/C_a670 is at least unity. The fact that the C_a670 maximum occurs as a shoulder on the short-wave slope of the red absorption band seems to justify this assumption.

(b) The shape of the C_a680 band is represented by the difference spectra of the crude preparations with a 2.5-min irradiation time. Support for this assumption is gained from the fact that, in nearly all cases, no shoulders around 670 nm showed up in these difference spectra. Moreover, the asymmetry of the half-width values (*i.e.* the proportional distances between wavelengths of maximum absorption and those of the short-wave and long-wave intersections for the half-width value and absorption band, respectively, expressed in % of the half-width equals that of the half-width value of chlorophyll *a* dissolved in methanol. The mean of 10 experiments gave values of 45 % and 55 % for the long-wave and short-wave parts, respectively, of the half-width values of the 2.5-min difference spectrum, whereas for chlorophyll *a* in the methanolic solution the values were 46 % and 54 %, respectively. It should be emphasized that this indication that the 2.5-min difference spectrum consists of only a single form is less convincing than the former observation on the shape of this spectrum.

(c) The shape of the C_a670 absorption band, and with it the asymmetry of the half-width value, is assumed to be the same as that of the C_a680 band. The shape of the absorption band obtained *in vivo* suggests that this approximation may be reasonable.

Based on these assumptions, a set of "spectra" was constructed by adding a series of percentages of C_a670 to C_a680. Next, the half-width values of these "mixtures" were determined, as well as the percentages due to C_a670 and C_a680 "absorption" at both 670 and 680 nm. The relation between the half-width value and percentage of C_a680 "absorption" at 680 nm and C_a670 "absorption" at 670 nm was then plotted. By determining the intensities around 670 and 680 nm of the absorption spectrum and the "normalized" difference spectrum, as well as the half-width value of the latter, the fraction of these bleached chlorophyll forms could be read from the graph.

For this construction of "mixed absorption bands", the spectra of C_a680 and C_a670 were approximated by triangles with the determined asymmetry of the half-width value. Since the exact shape of the component bands is unknown—for C_a680 the shape of the spectrum is only approximated—such a procedure seems useful for obtaining some insight into the reactions of the individual forms C_a680 and C_a670.

If unknown components overlap (Case 3), estimation of bleaching rates neces-

sarily becomes a rougher approximation than in the preceding cases. Nevertheless, an attempt was made to obtain some indication of a bleaching reaction of the C_a665 form. This was done by determining the half-width value of the difference bands and calculating the ratio: "absorption" of C_a670 at 665 nm to "absorption" of C_a670 at 670 nm as read from the constructed "absorption" bands mentioned for Case 2. Multiplication by this ratio of the amount of C_a670 bleached (determined according to the procedure of Case 2) yielded the approximate contribution of C_a670 bleached to the "normalized" difference band at 665 nm. This contribution was subtracted from the intensity of the "normalized" difference band at 665 nm, and the resulting value multiplied by n/a. The final value, then, may represent a rough approximation of the bleaching rate of C_a665. No correction for the overlap of chlorophyll *b* absorption was made. A discussion about the importance of these results is given below.

RESULTS

The aerobic photobleaching of the chlorophyll *a* forms C_a680 and C_a670 in crude and washed preparations is shown in Fig. 2. The bleaching of C_a680 in crude suspensions commences within 2.5 min of irradiation. The curves suggest that bleaching starts at once upon illumination. The same holds true for the washed preparations,

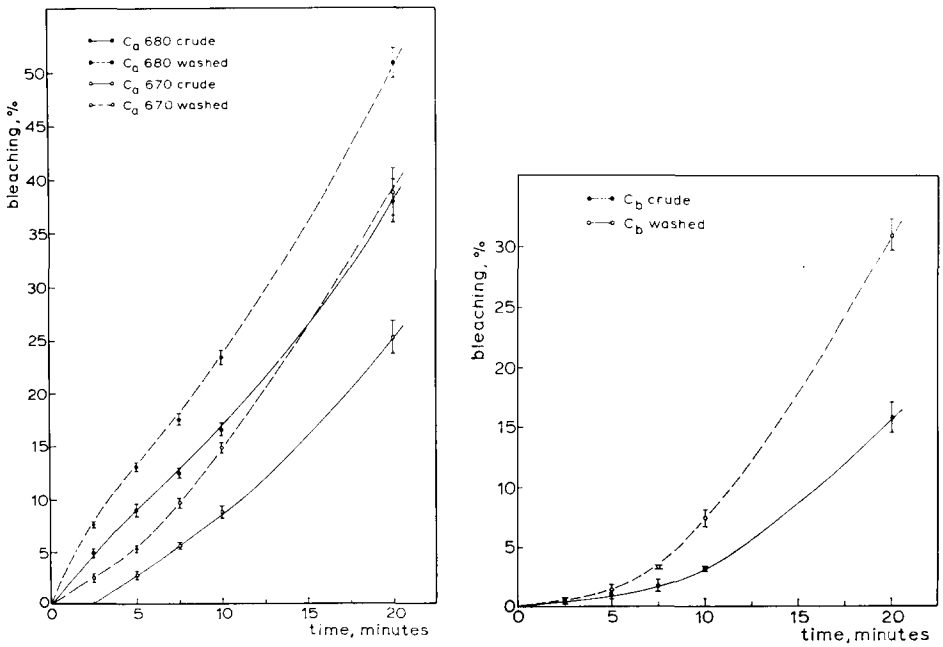

Fig. 2. Aerobic photobleaching of the major chlorophyll *a* forms in crude and washed preparations. The plotted values represent the means of 10 experiments. Their standard deviations are calculated according to the equation: s.d. $= \sqrt{\Sigma d^2/n(n-1)}$. The bleaching is expressed in % of the initial amounts for the individual forms. At the initial concentration, absorption at the mean maximum amounted to about 50%. Light intensity at the front window of the sample cuvette: about 0.3 W/cm^2.

Fig. 3. Aerobic photobleaching of chlorophyll *b*. For details see legend of Fig. 2.

albeit at an increased rate. In both cases, and during the first 10 min, bleaching increases approximately linearly with irradiation time.

On the other hand there is no appreciable bleaching of C_a670 in crude preparations for the first 2.5 min of irradiation. After that period, bleaching proceeds almost linearly with time up to 10 min, and at a lower rate than that for C_a680. Washing results in a distinct bleaching effect following a 2.5-min irradiation, whereas the curve suggests that bleaching starts immediately upon illumination. The increase of bleaching rate due to washing is somewhat higher for C_a670 than for C_a680 throughout the irradiation period.

The bleaching of chlorophyll *b* is given in Fig. 3. Bleaching is likely to start at once upon illumination, but its rate is much lower than that of C_a680 and C_a670, in particular for the first 10 min. Here again washing increases the bleaching rate. With both the crude and washed preparations a more or less exponential time course is found.

The bleaching increase with illumination time due to washing for C_a680, C_a670 and C_b is shown in Fig. 4. With C_a680, washing causes the bleaching to increase about 50%; the effect declines slightly with irradiation time. For C_a670 the effect drops from infinity to about 80%, and declines slightly with time too. For C_b the increase in question is about 50% for the first 5 min; it is increased to 80% after 7.5 min and continues increasing until a value of about 130% is reached after 10 min. It then declines somewhat faster than with the C_a forms.

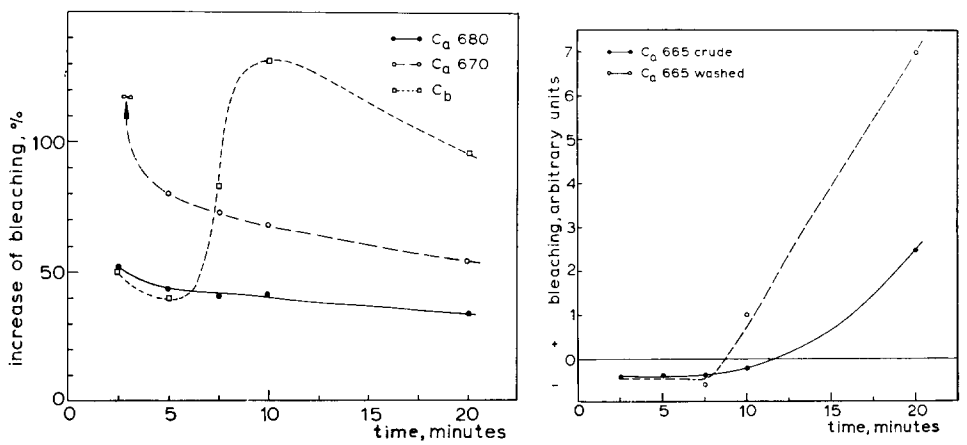

Fig. 4. Time course of the increase (%) in bleaching rate due to washing. The plotted values are calculated from the mean values plotted in Figs. 2 and 3. 0% refers to bleaching in crude preparations.

Fig. 5. Rough approximation of the aerobic photobleaching of C_a665. The plotted values are the means of 10 experiments. For further details see text.

An attempt was made to estimate the bleaching rate at 665 nm. As mentioned in PROCEDURE, such an estimation is inevitably only a rough approximation. It should be remembered that no correction was applied for the overlap of the C_b spectrum. Moreover, the absorption contribution due to the C_a forms may be over- or underestimated. In the case of absence of a C_a665 form one may assume (1) for correct estimation of the C_a absorption: bleaching at 665 nm follows the time course of C_b

bleaching, (2) for underestimation: the time course of bleaching at 665 nm is intermediate to those for C_b and C_a, and (3) for overestimation: the time course is intermediate to that for C_b and the inverse of that for C_a. The outcome of our approximation is depicted in Fig. 5. As the exact shapes of the C_b and C_a670 absorption bands are unknown, and these bands overlap that of C_a665 considerably, it is impossible to indicate the errors. For the same reason, the bleaching can only be expressed in arbitrary units. As Fig. 5 shows, negative values are obtained at shorter exposure times and, therefore, our approximation of the C_a670 absorption is exaggerated and the graphs are distorted. Still, they might suggest that (1) washing does not enhance bleaching at 665 nm for 7.5 min of irradiation, and (2) the bleaching increase due to washing after 20 min of exposure amounts to 180 %, whereas the sum of these increases for C_a670 and C_b (*cf.* Fig. 4) is 150 %. One may argue that the 30 % difference might be due to overlapping bleaching of C_a680. However, the fact that the 665-nm bleaching does not rise earlier than after 10 min of illumination seems to indicate that a C_a680 bleaching may be corrected for fairly well. Therefore, subject to many conditions and mainly because of the first-mentioned feature, it is suggested that a C_a665 form occurs, with bleaching properties different from those of C_a680, C_a670, and C_b.

Evidence for the existence of "short-wave" chlorophyll *a* forms other than C_a670 and C_a665 could not be obtained.

DISCUSSION

The light sensitivity of chlorophylls *in vivo* may be due, in the first place, to properties of the individual pigment–carrier complexes, *e.g.* redox potentials; secondly, to factors responsible for the effectiveness of energy transfer, such as the location of absorption and emission bands, concentration, and structure, or to both types of phenomenon. These factors may explain why the rates of photobleaching are different for various complexes. However, they cannot be solely responsible for differences in type of the respective time courses: *i.e.*, an approximately linear one for C_a680, a lag time followed by a more or less linear one for C_a670, the same type for C_a665 (except for a considerably longer lag period), and an exponential kind of time course for C_b. The effect of washing also differs for the various complexes, albeit that increase in bleaching rate occurred in all cases after prolonged exposure to light. Washing, therefore, seems to extract a protecting factor. W. TERPSTRA (unpublished results) obtained evidence that a DCIP-reducing factor is removed by washing.

It was checked in 5 experiments whether re-addition of the factor extracted from centrifuged crude preparation to sediments of washed preparations restored the bleaching rate to that of the original crude one. If the latter rate, measured after 20 min of irradiation, is taken as 100 %, the washed preparation showed a bleaching rate of 126 $\pm$ 3 %, and re-addition of the supernatant of the crude preparation to the sediment of a washed preparation restored this rate to 102 $\pm$ 1 %.

With C_a680, washing increased the bleaching rate by about 50 %. This rate declined slightly throughout the experiment. The decline, which was observed with C_a670 and C_b as well, may be real or artificial, or a combination of these possibilities. If artificial, it would be due to a slight underestimation, whereas in the former case the effect may be ascribed to a slow decrease in concentration of the protecting agent. As (1) a discrimination between these possibilities cannot be made with certainty,

(2) the decline in question is only slight compared with the changes in protection effectiveness to be discussed, and (3) it is probably not relevant with respect to the nature of the phenomena studied, this effect will be left out of further considerations.

At the high light intensities used, then, C_a680 bleaching starts upon irradiation and (for the range studied) proceeds at an approximately constant rate, thus indicating that the absorbed energy may not be drained quickly enough from the excited C_a680 molecules to prevent bleaching. According to DUYSENS (*cf.* ref. 14) this energy if transferred from the excited pigment molecules by inductive resonance *via* chlorophyll *a* to reaction centers. There are at least two chlorophyll *a* forms with different photochemical activities[15] and two photochemical systems, termed Systems 1 and 2 by DUYSENS, AMESZ AND KAMP[16]. C_a680 is considered to be involved in System 1, whereas its energy-trapping center is probably a form of chlorophyll *a* absorbing around 700 nm (*cf.* refs. 17, 18). This center, P700, occurs in a ratio of 1 in about 400 chlorophyll molecules (*cf.* ref. 19) and, according to KOK[20], its temporary bleaching at moderate light intensities is likely to be due to oxidation. Therefore, the removal of a reducing factor by washing may enhance its irreversible destruction in strong light. In its turn, the loss of trapping centers means increased instability for excited C_a680 molecules. On the other hand, depending on effectiveness of energy transfer between these molecules as well as light intensity, it may well be that, in addition, the C_a680 complexes are irreversibly bleached by photo-oxidation regardless of the presence of a trapping center.

The bleaching of C_a670 proceeds differently. The occurrence of a lag time in crude suspensions may be explained by assuming that there are trapping centers for System 2 which drain the excitation energy quite efficiently from the C_a670 complexes. It seems hard to explain the lag period in terms of a consumption of the protective factor, for if such were the case, a lag period would also be expected to occur with the bleaching of C_a680, and possibly C_b, in crude preparations. The absence of a lag time in washed preparations indicates that these centers are partially protected from photo-oxidation by the water-soluble factor mentioned. As soon as they are destroyed, either completely or in part, the bulk of C_a670 is open to photobleaching in a manner roughly similar to that for C_a680. At that moment the effect of washing resembles that for the latter chlorophyll form. Disregarding the slow decrease previously discussed, washing increases the bleaching rate of C_a670 in the same manner as for C_a680, though somewhat more intensively, namely by about 75 %. This line of reasoning suggests that the trapping centers for C_a680 (P700) are destroyed more readily by strong light than those for C_a670.

The problem of the System 2 trapping center has not yet been settled[21,22]. The occurrence of a lag time for C_a670 photobleaching in crude suspensions strongly argues in favor of the existence of such centers.

The fact that a lag period is observed for bleaching of C_a670 in crude preparations but not for C_a680 may be due to a greater stability of the C_a670 trapping centers as compared with those of C_a680; alternatively, their light sensitivity may be the same, but then the concentration of C_a670 centers should be higher than that of P700. The present experiments do not allow one to discriminate between these possibilities. It may be noted that the latter case may fit into the hypothesis of SAUER AND CALVIN[23] who suggested that a long-wave type of chlorophyll, C_a695, functions as a trap for C_a670. Since they assumed that the C_a695 concentration is about 5 %

of that of the total chlorophyll *a*, and this percentage for P700 is estimated[19] to be about 0.2 % whereas the amounts of C_a670 and C_a680 are nearly equal, it is implied that the System 2 trap concentration would be about 20-fold higher than that of System 1.

Chlorophyll *b* bleaches more slowly than the C_a types discussed. The shape of the time course of bleaching reminds one of an exponential phenomenon, and consequently seems indicative of a less complex situation than with C_a670 and C_a680. However, the effect of washing (*cf.* Fig. 4) suggests some kind of complication. It initially enhances the bleaching rate by about 50 %, but this increase is more than doubled around 7.5 min after the onset of illumination. This phenomenon indicates either that C_b may occur in at least two forms with different sensitivities towards removal of the protecting factor, or that some energy acceptor for excited C_b may undergo photodestruction to such a degree around 7.5 min of irradiation that an increase of the C_b bleaching rate results. Up to now, there has been no reason to suppose that this possible energy acceptor functions as a reaction center for some photochemical system. It might be, however, that some pigment facilitates or allows energy transfer from C_b to C_a670 and (to a smaller extent) to C_a680. Fig. 4 indicates that such a role, if it occurs, might be played by C_a665. Further experiments designed to discriminate between these possibilities are in progress.

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