THE RELATIONSHIP BETWEEN P-680 AND C-550

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ABSTRACT The published reports of flash-induced absorbance changes in the 680–690 nm spectral region, which have been attributed to bleaching of the primary reaction center chlorophyll of photosystem II (PSII) P-680, are discussed in light of what is known about the primary electron acceptor of PSII, C-550. The question of whether the fluorescence yield changes, which accompany the photoreduction of C-550, might influence the measurements of chlorophyll bleaching is examined. The responses attributed to P-680 and their relationship to C-550 indicate that, if the absorbance measurements are valid, P-680 probably functions as the primary electron donor to PSII rather than as a photochemical sensitizer of the primary redox reaction.

INTRODUCTION

Two light-induced absorbance changes have been identified with the primary photochemistry of PSII. A bleaching at 690 nm was attributed to the reaction center chlorophyll of PSII by Döring et al. (1, 2). The same reaction was studied at −196°C by Floyd et al. (3) who labeled the pigment P-680 by analogy with the P-700 associated with PSI. Floyd et al. considered P-680 to be the primary electron donor to PSII while Döring et al. considered the pigment to be a sensitizer of a primary photochemical electron transfer reaction but not to be involved directly with a redox change. Another light-induced bleaching was shown by Knaff and Arnon (4) to occur near 550 nm at −196°C as well as at room temperature. They attributed the absorbance change to a photochemical reduction of a component, C-550, by PSII. Erixon and Butler (5) showed that there was one-to-one correspondence between the redox changes of C-550 and the redox changes of Q, the fluorescence quencher and presumably the primary electron acceptor of PSII. The present note examines the relationship between P-680 and C-550 in the hope that such an examination will provide insight into the primary photochemistry of PSII and elaborate points of controversy or uncertainty which may be subjected to experimental resolution.

The measurement of P-680 is complicated by fluorescence yield changes because fluorescence excited by the measuring beam may be a detectable part of the meas-

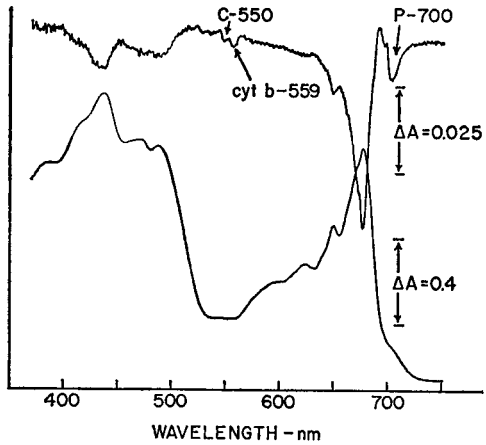

FIGURE 1 Absorption spectrum of a sample of chloroplasts (20 μg chlorophyll/1.1 cm²) frozen to −196°C and the difference spectrum (irradiated minus dark) due to irradiation with red light at −196°C. The spectra were measured with a single-beam spectrophotometer on line with a PDP/8I computer (5).

ured signal. This artifact, as was pointed out previously (6), is particularly troublesome because the light-induced increase of fluorescence yield results in changes that resemble a photobleaching of chlorophyll. Fig. 1 shows the absorption spectrum of a sample of chloroplasts at −196°C and the difference spectrum induced by irradiating that sample with red light. Irradiation at −196°C oxidizes P-700 and reduces C-550. (The photooxidation of cytochrome [cyt] *b*-559 which accompanies the photoreduction of C-550 [5] is also apparent.) The relatively large absorbance change of P-700 is due to the intensification of absorption by light scatter which occurs in highly scattering samples in spectral regions of low absorbance (7). Photoreduction of C-550 causes an increased fluorescence yield so that the measuring beam, as it scans through the chlorophyll and carotenoid bands, produces more fluorescence thus giving an apparent bleaching of those bands. The fluorescence yield artifact can be eliminated readily in the blue region by using a blocking filter which does not transmit the fluorescence, but it is not possible to eliminate all fluorescence from measurements in the red.

The increased fluorescence yield is stable at −196°C. At room temperature the increased yield induced by light decays in the dark. Much of the evidence for P-680 has come from measurements of light-induced absorbance changes by repetitive flashes (1, 2, 8). Such measurements will be affected only by that portion of the variable fluorescence yield which decays during the dark period between flashes. Forbush and Kok (9) attempted to measure the decay of fluorescence yield after a single brief flash when the pool of redox components (labeled *A* pool) was largely oxidized. They determined a half-time of 0.6 msec for the decay of fluorescence yield which should probably be considered an upper limit because of the time con-

stants in their measurements. If fluorescence yield changes were responsible for the bleaching ascribed to P-680, the fluorescence yield would have to decay with a half-time of 0.2 msec since that is the half-time for the recovery of the P-680 at room temperature (1, 2). The available data do not rule out the possibility of such a fast decay.

The magnitude of the fluorescence yield artifact will depend on the density of the sample, the fluorescence yield and fluorescence yield change of the chlorophyll, the fraction of the fluorescence collected by the phototube, and the light-scattering properties of the sample. With an optically clear sample, fluorescence could be eliminated by using a parallel measuring beam and placing the phototube at sufficient distance behind the sample. With light-scattering samples such a solution has practical limitations; e.g., no advantage would be gained by limiting the solid angle of light collection if the sample were sufficiently scattering to completely diffuse the measuring beam.

In the instrument used by Döring et al. the phototube was 1 m behind the sample and a lens was used to focus the exit slit of the monochromator on a 0.5 × 1.5 cm slit at the phototube (G. Döring, personal communication). This optical arrangement should minimize fluorescence as much as possible; however, some question still remains, even with very small solid angles, of how effectively fluorescence can be excluded from the light transmitted by a light-scattering sample.

A strong argument in favor of the validity of the P-680 measurements is that the absorbance changes attributed to the Soret band of P-680 at 435 nm have the same kinetics and have the same dependence on heat treatment (2, 10) as the changes in the red. Until the fluorescence yield artifact is experimentally ruled out, however, there is the possibility that the changes in the blue are due to a still undiscovered Soret band of C-550 and that the changes in the red, which would follow precisely the kinetics in the blue, are due to the fluorescence yield changes which accompany the redox changes of C-550.

It may be noted that the fluorescence yield artifact in Fig. 1 gave a maximal bleaching at the chlorophyll absorption maximum, as should be expected, while the difference spectra reported by Döring et al. show maxima which are at slightly longer wavelengths than the absorption maxima, e.g., 690 nm for normal chloroplasts (1) and 682 nm for the PSII-enriched particles (2). The use of interference filters as guard filters at each of the measuring wavelengths, however, would tend to shift the apparent bleaching due to fluorescence to a longer wavelength.

The previous discussion may have belabored the fluorescence yield artifact to an unreasonable extent in view of precautions employed by Döring et al. Döring (10) estimated that the fluorescence excited by the measuring beam, even without a guard filter, was too low to be a detectable part of the bleaching signal so that with a guard filter, which reduces the fluorescence by approximately an order of magnitude, the fluorescence artifact should be truly negligible. A direct experimental test would be

even more convincing, however. If the 690 nm guard filter could be removed without complications from the flash artifact, the question of the fluorescence yield artifact could be settled directly. If removing the guard filter had no influence on the bleaching signal, which Döring's estimate of fluorescence would predict, the fluorescence artifact could be ignored. If, on the other hand, the bleaching increased when the guard filter was removed, comparing results obtained with guard filters which transmitted differing amounts of fluorescence would establish the extent to which fluorescence was a problem.

Assuming that the absorbance measurements at 680–690 nm are valid, the comparison of P-680 and C-550 may be focused on how these components might interact and whether P-680 acts as a sensitizer of the primary photochemical reaction of PSII or as the primary electron donor to that reaction. Let us also examine the effects of different reagents and treatments on P-680 and C-550 in an effort to find conditions where P-680 changes are inconsistent with the changes of fluorescence yield. The comparison between P-680 and C-550 will depend heavily on the hypothesis, which has been reasonably well established (5), that C-550 is the primary electron acceptor of PSII (or is an excellent indicator of the redox state of the acceptor) and is isomorphic with Q, the fluorescence quencher of PSII.

3-(3,4-DICHLOROPHENYL)-1,1-DIMETHYLUREA (DCMU)

Döring et al. (1) found that DCMU eliminated the reversible absorbance change of P-680 in the same concentration range that inhibits oxygen evolution. They concluded that DCMU reacts directly at P-680 (chlorophyll [Chl] a_{II} in their terminology) to eliminate the primary photochemical reaction of PSII; however, DCMU does not block the photoreduction of C-550, either at room temperature (4) or at -196°C (unpublished results from our laboratory). Rather, it seems to block the dark oxidation of reduced C-550 (4). The apparent anomaly of how DCMU could block the P-680 change but not the C-550 change is resolved, however, by noting that the intensity of the measuring beam was sufficient to reduce C-550 in the presence of DCMU. In that case there will be no fluorescence yield artifact because the fluorescence yield is maximal and cannot be increased further by the flash. Furthermore, the photooxidation of P-680, if it were the primary electron donor to PSII, would probably be blocked when C-550 is reduced because of the absence of an electron acceptor. Erixon and Butler (5) showed that the photooxidation of cyt *b*-559 by PSII at low temperature did not occur when C-550 was in the reduced state, apparently because the strong oxidant of PSII is not formed photochemically when the primary electron acceptor is not available.

HEAT TREATMENT OR TRIS WASHING

Döring et al. (2) found that the P-680 change survived in heat-treated chloroplasts and Govindjee et al. (8) found the same in Tris-washed chloroplasts even though

oxygen evolution was destroyed in both. They interpreted the correlations that the P-680 change did not need electron transport but electron transport did need the P-680 change (from the DCMU data) to indicate that P-680 was a sensitizer of the primary electron transfer step but did not participate directly in a redox change. Heat treatment and Tris washing block electron transport between water and PSII but leave PSII intact as shown by PSII activity when artificial electron donors are added (11). Under steady irradiation the fluorescence yield of Tris-washed chloroplasts increases very little because there are not sufficient endogenous electron donors to reduce the pools of redox compounds which can oxidize C-550 (cf. reference 12). In the presence of DCMU, which blocks electron transport immediately after C-550, the fluorescence yield does increase to its maximal level during irradiation (12) showing that the endogenous donor is able to reduce C-550. With repetitive flash excitation we would expect a rapid reduction of C-550 and oxidation of P-680, and restoration of these components to their original states during the dark period between flashes. Since cyt *b*-559 can be oxidized by PSII in Tris-washed chloropasts (13, 14) a cycle around PSII might operate via cyt *b*-559. Such a cycle would also act to keep the fluorescence yield low during irradiation in steady irradiation. Thus, the heat-treated or Tris-washed chloroplasts do not militate conclusively against the fluorescence yield artifact nor do they argue strongly for the sensitizer role for P-680.

HEPTANE EXTRACTION

Govindjee et al. (8) found that heptane extraction of dry lyophilized chloroplasts destroyed the P-680 change. The extraction also eliminated most of the light-induced fluorescence yield increase since the fluorescence yield of the chloroplast sample remained near its normal minimum level even during irradiation (15). When the heptane extraction was carried out in the presence of a trace of water, however, the P-680 change remained while most of the fluorescence of variable yield was lost. In this case the minimum fluorescence yield was increased, approaching the maximum level. Thus, the "wet" heptane-extracted chloroplasts may provide a system where the P-680 changes occur in the absence of the fluorescence yield changes. At least, this system should be explored further.

Floyd et al. (3) measured rapid absorbance changes in chloroplasts at $-196°C$ induced by a single flash. At 680 nm the flash caused an instantaneous bleaching followed by a partial recovery with a half-time of about 10^{-2} sec in spinach chloroplasts which was essentially independent of temperature from $-50°C$ to $-196°C$. At 700 nm a bleaching occurred because of the photooxidation of P-700, which did not recover in the dark. (The very rapid partial recovery of the bleaching, 30 μsec half-time, observed at both 680 and 700 nm could have been caused by the decay of luminescence excited by the flash.) The 10^{-2} sec recovery phase of P-680 was correlated with an oxidation of cyt *b*-559 which indicated that P-680 was the primary electron donor to PSII and that the oxidized P-680, in turn, oxidized cyt *b*-559, at

least at low temperature. The measurements of P-680, i.e. the recovery of absorbance after the bleaching, by Floyd et al. (3) were about 100 times greater than those of Döring et al. (1) (ΔA of 6×10^{-3} vs. 4×10^{-5}) even though the samples were comparable in terms of chlorophyll. Somewhat greater values should be expected from samples frozen to −196°C because of the increased path length due to light scatter, but that effect should be rather small at 680 nm where the density of the sample is high (7).

The extent to which the low temperature measurements of P-680 by Floyd et al. may have been confounded by fluorescence yield changes is uncertain. A red cutoff filter was used to block the actinic flash from the phototube but no mention was made of using a 680 nm interference filter to minimize fluorescence. From what is known about the fluorescence yield changes at −196°C induced by continuous irradiation, the increased fluorescence yield induced by a flash at −196°C would not be expected to decay in the dark after a flash. It is possible, however, that electron transport occurs one step beyond C-550 at low temperature and that until that next pool is reduced, partial oxidation of C-550 could occur in the dark after a flash. The correlation between the rate of cyt *b*-559 oxidation and the rate of recovery of P-680 can be taken as evidence that the 680 nm absorbance changes are not fluorescence yield artifacts since there is no reason to expect the decay of the fluorescence yield to have the same kinetics as the oxidation of cyt *b*-559. The significance of the fluorescence yield artifact, however, could probably be established more firmly by direct experimental tests.

The evidence implicating the fluorescence yield artifact is largely circumstantial. The chances of a significant fluorescence yield artifact would appear to be greater in the measurements by Floyd et al. (3), since precautions were not taken to block fluorescence from the phototube and it is in these measurements that the bleaching at 680 nm is greatest. The bleaching measured by Döring et al. was extremely small. We calculate one P-680 per 10^4 chlorophylls from their data (1) assuming complete bleaching of the P-680 absorption band and equal extinction coefficients. (We believe their value of 1:1000 [1] must have included a calculation error.) Furthermore, practically all of the known responses of P-680 to inhibitors like DCMU or to treatments such as Tris washing or heating could be accounted for by the fluorescence yield change if it were detectable. Only the heptane extraction with a trace of water present seemed to give a condition where the fluorescence yield changes would not account for the P-680 changes. As such, that treatment should be explored further; however, as indicated above, it should be possible to rule out the fluorescence yield artifact by a direct experimental test if it does not play a role.

If the measurements of P-680 are shown to be valid, the question of whether P-680 acts as a sensitizer of the primary photochemical reaction or participates as the primary electron donor seems to be resolved in favor of the latter hypothesis. At least the reasons for invoking the sensitizer role can be accommodated as well by the donor role whereas the reverse is not true.

In either event the responses of P-680 and C-550 are closely intertwined. In the case of the fluorescence yield artifact, P-680 bleaching is merely a reflection of the photoreduction of C-550. If P-680 is the primary electron donor to PSII, its photoresponses should be complementary to the responses of the primary acceptor, C-550, because photooxidation of the donor must be accompanied by the photoreduction of the acceptor. Other close correlations should also be found. For example, chemical reduction of C-550 should block the photoreduction of P-680. Mutants which lack C-550, of which there are several in *Chlamydomonas* (16) and *Scenedesmus* (17), should not show the P-680 change.

The proposition that the rapid, flash-induced 680 nm absorbance changes represent redox changes of the primary electron donor and that the light-induced absorbance changes near 550 nm and the fluorescence yield changes represent redox changes of the primary electron acceptor of PSII provides a promising experimental framework. If flash-induced absorbance changes of C-550 could be measured, the kinetics of the recoveries of C-550 and P-680 after the flash should separate the two processes and provide information about the secondary electron transport reactions. A study correlating the kinetics of the decay of the fluorescence yield and the dark oxidation of C-550 after a flash might provide additional evidence supporting the isomorphic relationship between Q and C-550 or give important information as to their differences. It is hoped that this examination of P-680 and C-550 will stimulate controversy, insight, and research efforts which will further elucidate the primary photochemical mechanisms of photosynthesis.

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