Stabilization of Charge Separation and Photochemical Misses in Photosystem II†

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ABSTRACT: Illumination of photosystem II by a saturating short flash results in a stabilized charge separation in only about 90% of the reaction centers. During a series of flashes, the 10% fraction of "photochemical misses" is randomly redistributed among the centers. This phenomenon is investigated in DCMU-inhibited material, eliminating the contribution to misses due to electron transfer equilibrium on the quinone acceptors. Under such conditions, the miss coefficient is about 5% and is enhanced to about 40% in the presence of hydroxylamine at low pH. When a second flash is fired, its efficiency increases as a function of the time delay after the first flash (turnover experiments). This process involves three distinct time domains: <10 μs, 100 μs, and 10 ms. From a study of the 515-nm field-indicating change, it appears that the increased inefficiency caused by hydroxylamine is not due to a lesser amount of initial charge separation but to a recombination process concomitant with the 100 μs phase of the turnover. The slow turnover phase (10 ms) is not associated with a recombination or any other electron transfer event but reflects a modification of open centers during which their probability to achieve charge stabilization rather than recombination is progressively increased. These results are interpreted in terms of an equilibrium between two conformational states of the centers endowed with different stabilization yield ("good" and "bad " stabilizers). The 100 μs turnover phase is due to the reopening of the bad stabilizers by recombination, and the 10 ms phase accompanies the redistribution of these centers among the two conformational states.

When chloroplasts are subjected to a sequence of short saturating flashes, the yield of the flash-induced oxygen evolution displays marked oscillations with a periodicity of four flashes, as originally discovered by Joliot and co-workers (*1*). This reflects the requirement for accumulation of four oxidizing equivalents on the PS II[1] donor side in order to oxidize two water molecules and form molecular oxygen. A striking feature in such experiments is the damping of the oscillatory pattern. This damping was explained by Kok et al. (*2*) by assuming the occurrence of two probabilistic phenomena. First, a fraction of the centers appears unable to achieve charge separation (photochemical misses). This fraction is on the order of 10%. Second, some other fraction undergoes two charge separations (double-hits). The latter phenomenon is artifactual in the sense that it can be suppressed by using sufficiently short flashes, such as submicrosecond laser flashes. The photochemical misses, on the other hand, reflect an intrinsic mechanism. The probabilistic character should be emphasized in the first place: the fraction of centers that fail in the photochemical process is not a permanent (heterogeneous) population, but must be more or less randomly distributed upon each flash in order to account for the damping (*3*).

As pointed out by Joliot and Kok (*3*), there are two, nonexclusive, possibilities that may account for the misses. The first one assumes that a fraction of the centers happens to be photochemically inactive when the flash is fired. This may be the case if there is an equilibrium between open and closed conformations of the center. The PS II center is endowed with charge accumulation systems both on its donor and acceptor side, so that redox equilibria with an oxidized secondary donor or reduced secondary acceptor may cause a fraction of centers to be in the closed $P_{680}^{+}\,Q_A$ or $P_{680}\,Q_A^{-}$ states, respectively. This view was developed by Shinkarev and Wraight (*4*) in their "bicycle" model, predicting specific oscillating patterns for the miss probabilities caused by the donor and acceptor side equilibria. We would like to briefly discuss this model. On the acceptor side, the secondary quinone Q_B will be predominantly in the semiquinone state Q_B^{-} after each odd-numbered flash and in the oxidized Q_B state after even flashes. The equilibrium constant with the primary quinone Q_A, $[Q_AQ_B^{-}]/[Q_A^{-}Q_B]$ has been estimated to be about 20 at pH 7 and larger at lower pH (*5*−*7*). Hence, about 5% of the centers are expected to be in the closed state $P_{680}\,Q_A^{-}Q_B$ when an even-numbered flash is fired. This equilibrium occurs on every other flash, and thus entails an average miss probability of 2.5%, or less at pH <7 [e.g., 1% at pH 6.5, taking the equilibrium constant estimated by Crofts et al. (*7*)]. Therefore, this process cannot account for the totality of the observed misses. This is supported by the fact, which will be scrutinized in this paper, that misses are still observed in the presence of an inhibited acceptor side. Shinkarev and Wraight also proposed that a similar mechanism occurs on the donor side via equilibria involving states S_2 and S_3 of the oxygen-evolving complex: $S_{2,3}P_{680} \rightleftharpoons S_{1,2}P_{680}^{+}$. We believe, however, that the relevant

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[1] Abbreviations: DCMU, 3-(3,4-dichlorophenyl)-1,1-dimethylurea; PS II, photosystem II.

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equilibrium constants are likely too large to result in a significant fraction of P_{680}^+. The half-time for the recombination of $P_{680}^+Q_A^-$ is about 120 μs (*8*) (or, according to ref *9*, 1 ms). On the other hand, the half-time for the recombination of $S_2Q_A^-$ is 1−2 s so that the equilibrium constant $[S_2P_{680}]/[S_1P_{680}^+]$ lies in the 10^3-10^4 range. A similar figure is expected for the equilibrium involving S_3 (*10*). Therefore, equilibria on the donor side can hardly account for a contribution to the miss probability greater than 0.1%. Shinkarev and Wraight arrived at a different conclusion by compiling literature data for the Y_ZP equilibrium (K_{YP} = 2−5 derived from ref *11*) and for the $S_{2,3}Y_Z$ equilibrium ($K_{2,3Y}$ = 5−9; *12*). The former equilibrium constant was estimated from the relative amplitude of the 35 μs phase of P_{680}^+ reduction. It should be noticed, however, that this phase cannot reflect relaxed equilibrium conditions (which are relevant here), since it does not match the kinetics of Y_Z^+ reduction. This is particularly obvious for the $S_3Y_Z^+$ state where a 35 μs phase of P_{680}^+ is still observed whereas the decay of Y_Z^+ occurs in the millisecond time range. Therefore, the 35 μs phase of P_{680}^+ reduction can either be due to a relaxation process or, as will be argued later, to a conformational heterogeneity. Conversely, the fact that phases of P_{680}^+ reduction that would kinetically match the S state-dependent rates of Y_Z^+ decay have not been resolved is in favor of a fairly large equilibrium constant at this step.

An alternative (non-exclusive) model for the misses does not resort to the presence of closed centers but to a probability of recombination occurring before the separated charges are stabilized. All open centers undergo a photochemical reaction, but a fraction of these might revert to the initial state due to the competition between recombination and stabilization in, say, the $S_2Q_A^-$ state. The present paper reports evidence for such a process and gives an insight on the kinetic aspects involved in this mechanism. The results suggest that a conformational equilibrium controls the recombination/stabilization competition.

MATERIALS AND METHODS

Most of the experiments were performed with whole cells of the green alga *Chlorella sorokiniana* as described in refs *13* and *14*. We used the mutant strains S-11 and S-56 (kindly provided by Dr. P. Bennoun), which are devoid of the photosystem I reaction center. The double mutant S-56 is also lacking LHC II and was used for measurement of the C-550 absorption change. To inactivate the metabolic activity and maintain the plastoquinone pool oxidized in the dark-adapted state, the cells were treated with benzoquinone as follows. The algal culture was incubated with 200 μM *p*-benzoquinone (sublimed before use) during a 10 min centrifugation. The pellet was then resuspended in a benzoquinone-free medium, containing 50 mM KCl and 100 mM buffer (MES for pH < 7 or HEPES for pH > 7). Spinach thylakoids were prepared as in ref *15*. Absorption changes were measured with the Joliot-type apparatus (*16, 17*), using monochromatic flashes (2 μs duration) as a detecting beam. Fluorescence was measured with the same setup, as described in ref *15*. Several types of actinic flashes were used as indicated: (i) A xenon lamp (flash duration: 2 μs at half-height) using a broad band red filter for absorption change experiments or a broad band blue filter for fluorescence measurements. (ii) A Q-switched ruby laser (695 nm) with 50 ns pulses. (iii) In the fluorescence experiment of Figure 2, we also used a Nd:YAG laser (frequency doubled, 532 nm), with 6 ns pulses. In the turnover experiments, the first flash was a ruby laser pulse, followed after a variable delay by the xenon flash. The samples were pumped into the apparatus cuvette from a dark vessel and discarded after each measurement when hydroxylamine was present or, when only DCMU was present, pumped back into the reservoir with a mean cycling time of about 100 min.

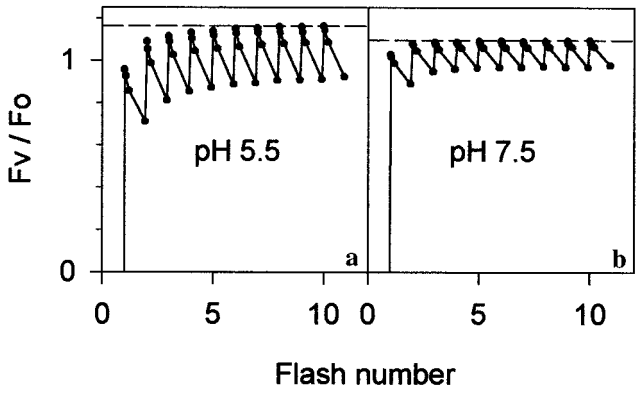

FIGURE 1: Changes of the chlorophyll fluorescence yield during a series of saturating xenon flashes in DCMU (10 μM)-poisoned algae (S-11) incubated at pH 5.5 (a) or pH 7.5 (b). The fluorescence was sampled at 1, 5, 25, and 115 ms after each flash (spaced 125 ms apart). The vertical scale is the variable fluorescence (F_v) normalized to the dark-adapted level (F_o). The dashed lines indicate the asymptotic levels.

RESULTS

The experiment of Figure 1 shows the changes of the fluorescence yield induced in DCMU-poisoned algae (S-11) by a series of saturating, short (2 μs at half-height) xenon flashes. The fluorescence yield reflects in a nonlinear way the amount of closed centers in the Q_A^- state. Due to the presence of DCMU, inhibiting the Q_A to Q_B electron transfer, the reoxidation of Q_A^- occurs in the seconds time range (through recombination with S_2), so that the photoinduced fluorescence change can be measured conveniently in the millisecond range. The low ratio (≈1) of the maximum variable fluorescence F_v over the dark fluorescence level F_0 observed with S-11 is due to the fixed fluorescence contribution of the PS I antenna, which is normally small when the PS I reaction center is present. Despite the saturating character of the flash, the level reached after the first flash is below the maximum level attained after several flashes. The fluorescence yield measured at 1 ms after the first flash corresponds to 95% (panel b, algae incubated at pH 7.5), or 90% (panel a, algae incubated at pH 5.5) of the maximum variable fluorescence. This effect of pH (also apparent in the faster recombination rate at pH 5.5) suggests that in benzoquinone-treated algae the chloroplast equilibrates with the pH of the medium. Taking into account the hyperbolic relation between the fluorescence yield and $[Q_A^-]$ (*18, 19*), the true amount of photochemical misses is 2−3-fold smaller than suggested by these figures, i.e., about 2% at pH 7.5 and 5% at pH 5.5. This estimate of the misses in the presence of DCMU is relative to centers that are open (Q_A oxidized) before the flash. As explained in the Introduction,

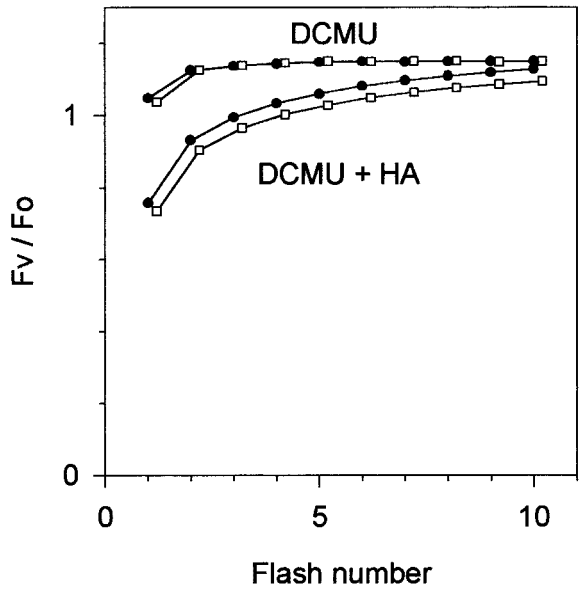

FIGURE 2: Fluorescence level sampled at 1 ms after each flash of a series, using either a xenon flash (closed circles) or a frequency doubled Nd:YAG laser (open squares; the symbols were slightly shifted to the right for clarity). Both types of flashes were saturating and spaced 150 ms apart. The algae were incubated at pH 6 in the presence of DCMU (10 μM) or DCMU and hydroxylamine (2 mM) as indicated. The lines were drawn to connect the 1-ms data point following each flash and do not represent the actual time course of the fluorescence change (at variance with, for example, Figure 1).

in the noninhibited system, the additional contribution due to the $[Q_AQ_B^-]/[Q_A^-Q_B]$ equilibrium would take place.

On subsequent flashes, the fluorescence level rises toward an asymptotic level. This process can be examined more accurately at pH 5.5 than at pH 7.5, due to the larger miss coefficient. The slow rise observed beyond the second flash is not consistent with the ca. 95% efficiency estimated for the first flash but rather with a yield of about 50%, reflecting a small population of centers (about 5%) with a low charge separation efficiency.

Similar results were obtained with isolated spinach thylakoids (not shown, but see ref *23* or *31*), except for the extent of the F_v/F_0 ratio, which is 3−4 in stacked thylakoids.

Figure 2 shows a comparison between the successive fluorescence levels observed during a series of xenon flashes or Nd:YAG flashes (6 ns duration). The observed pattern is very similar in both cases. In particular, the level reached after the first flash was only slightly diminished when using the laser flash. This result was also obtained (see below) with a Q-switched ruby laser, delivering 50 ns pulses. Hence, in agreement with ref *20*, we found no confirmation of the finding by France et al. (*21*) that the fluorescence change is markedly smaller when using a sub-microsecond flash (this is expected to occur, however, with picosecond flashes, when singlet−singlet anihilation becomes significant).

We now address the question of the turnover of the misses, i.e., how the increment of the fluorescence yield caused by the second flash depends on the time interval between the two flashes. In the experiment of Figure 3, the first flash was a ruby laser pulse (50 ns duration) followed after a variable delay by a xenon flash (both saturating). The fluorescence yield reached after the second flash was plotted as a function of this delay. Figure 3 shows the results obtained with cells incubated at pH 7.5 or pH 5.5, plotted on a short (panel a) and long (panel b) time scale. The increase of the yield caused by the second flash rises with multiphasic kinetics. The outcome of a three-exponential fit is indicated in the legend. A first phase, which is not very accurately resolved (because of the shape and duration of the xenon flash) occurs in the few microseconds time range. A second phase takes place in the hundreds of microseconds range and a slow one in the tens of milliseconds. At longer times, a decrease was observed, due to the recombination reaction that progressively resets the system into its initial state.

The inefficiency of the flash-induced charge separation can be dramatically increased by adding hydroxylamine (*22, 23*). The effect of this substance is illustrated in Figure 4 (panel a), and its pH dependence is shown in panel b, using spinach thylakoids. Similar results were obtained with algae, as shown below (and in Figure 2). Hydroxylamine destroys the Mn cluster of the oxygen-evolving complex and serves as an artificial electron donor to Y_Z (*24*). It also causes a dramatic slowing down of the rate of P_{680}^+ reduction by Y_Z, like other treatments (e.g., Tris washing) that destroy the Mn cluster (*25*). The lifetime of P_{680}^+ is thus shifted from the 30−200 ns range in the native system to the microsecond range. As shown by Conjeaud and Mathis (*8, 26*), the reaction with Y_Z in such systems is markedly pH-dependent: the half-time of P_{680}^+ decay is about 2 μs at pH 8 and about 15 μs at pH 5. The effect of pH shown in Figure 4 is presumably linked to the longer lifetime of P_{680}^+ at low pH, entailing a greater probability for charge recombination.

The experiments of Figure 5 (panel a) analyze the turnover kinetics in the presence of DCMU and hydroxylamine. Due to the increased flash inefficiency, the amplitude of the fluorescence rise caused by the second flash is larger than in the absence of hydroxylamine, and the kinetics are better resolved. Again, multiphasic kinetics are found, with time constants in the microseconds, hundreds of microseconds, and tens of milliseconds domains (see the fit results in the legend). It is noteworthy that the recovery of the second flash efficiency occurring during the slow (tens of milliseconds) phase is not accompanied by a significant change of the fluorescence level, as shown in panel b. This level remains indeed almost constant at times longer than about 1 ms, while at shorter times a rise of the yield was observed (not shown), likely reflecting slow phases of P_{680}^+ reduction. Thus, the increase of the photochemical efficiency observed in the tens of milliseconds range is not due to a reopening of the centers through reoxidation of Q_A^-.

The important miss probability occurring in the presence of hydroxylamine offers an opportunity to study this phenomenon using flash-induced absorption changes. This technique is less sensitive than fluorescence but gives useful additional insight. Figure 6 shows a comparison of the field-indicating change observed at 515 nm after the first flash in DCMU-poisoned algae, in the absence or presence of hydroxylamine. The field-indicating change (C-515 or carotenoid shift) is an electrochromic signal due to the spectral shift of light-harvesting pigments (mostly carotenoids), responding linearly to the membrane potential. The initial amplitude of this change reflects the amount of charge separation (strictly due to PS II in a PS I-lacking mutant). At short times, there is an interference from the large

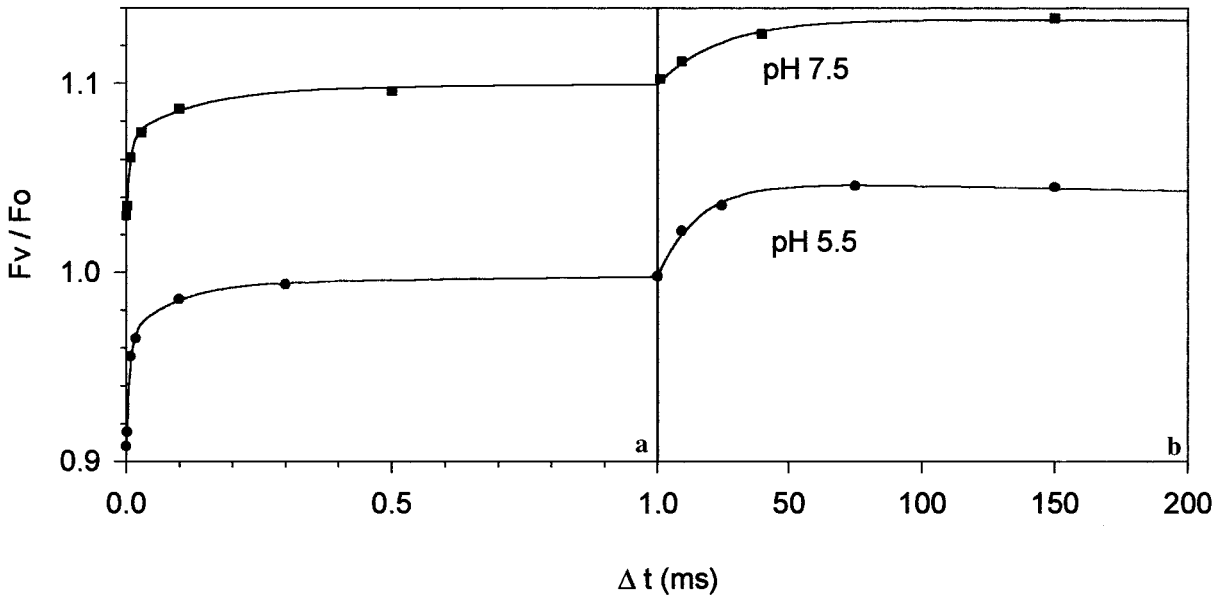

FIGURE 3: Turnover experiment in DCMU-poisoned algae incubated at pH 5.5 or pH 7.5. The data points indicate the fluorescence level measured at 1 ms after a second (xenon) flash, as a function of the time delay between this flash and a first one (ruby laser). The data are displayed using a short (panel a) and long (panel b) time scale. The first data points correspond to $\Delta t = 0$ (no xenon flash), 2, 8, 18, 100 μs ... (taking approximately into account the width of the xenon flash). The curves indicate the results of an adjustment with a sum of three exponential components (and a slower decay), yielding $t_{1/2}$ and relative amplitudes as follows: 4.5 μs (42%), 64 μs (20%), and 11 ms (38%) at pH 5.5; 4.9 μs (41%), 92 μs (25%), and 16 ms (34%) at pH 7.5.

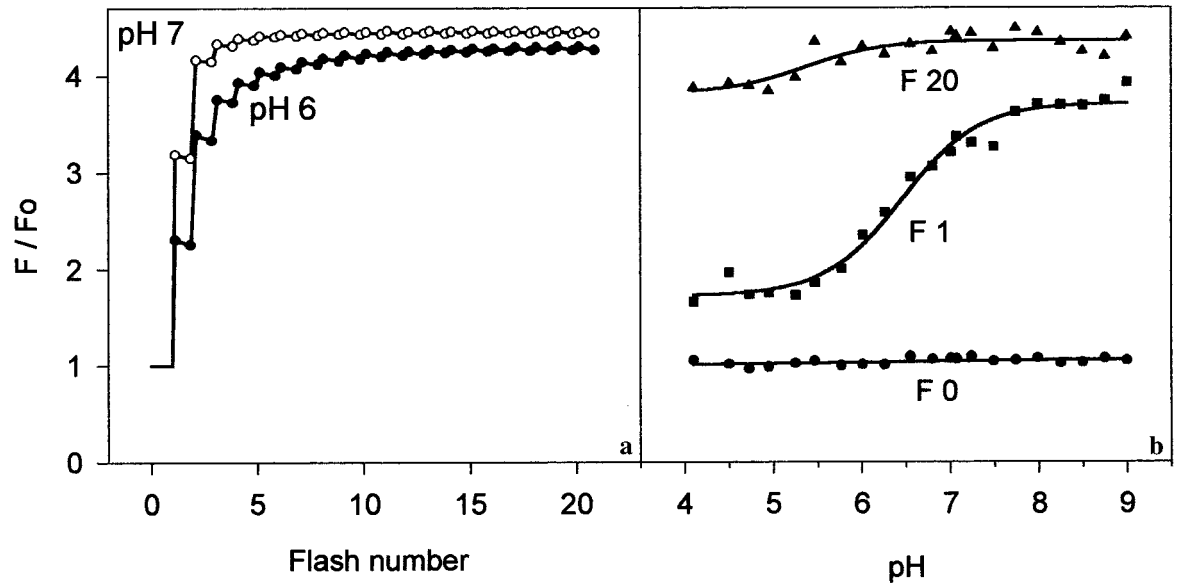

FIGURE 4: Changes of the fluorescence yield induced by a series of saturating xenon flashes in spinach thylakoids in the presence of DCMU (10 μM) and hydroxylamine (2 mM). Panel a shows the sequences observed at pH 6 and pH 7 (fluorescence sampled at 1 and 150 ms after each flash; flash spacing 200 ms). Panel b shows a plot of the dark-adapted level F_0, the 1-ms levels reached after the first (F1) and 20th flash (F20) as a function of pH.

absorbance change caused by the flash-induced formation of ^{T}Car (*27*−*29*), which decays with $t_{1/2} \approx 5$ μs. The field-indicating change can be reliably measured from 50 μs onward, and its initial extent may be obtained by extrapolation to time zero. The subsequent decay of the signal monitors the collapse of the membrane potential caused by ionic transport across the membrane or by reversal of the charge separation (recombination). In the experiment of Figure 6, in the absence of hydroxylamine, the decay of the membrane potential in the 50 μs−10 ms range is about 10% of the total change. In the presence of hydroxylamine, the initial charge separation is about the same, but a decay phase of about 30% (at pH 6) is occurring, with $t_{1/2} \approx 150$−200 μs. It thus appears that the inefficiency of the flash-induced charge separation caused by hydroxylamine does not correspond to a diminished initial amount but to a recombination reaction occurring in the 100 μs range. This view is supported by measurements of the absorption change difference (540−550 nm), monitoring the formation and decay of Q_A^- (C-550). This experiment (not shown) was run with the double mutant S-56, which like S-11 is devoid of the PS I reaction center and is also depleted in LHC II, so that the above wavelength difference is only little contributed by the field-indicating change. The initial amount of Q_A^- measured

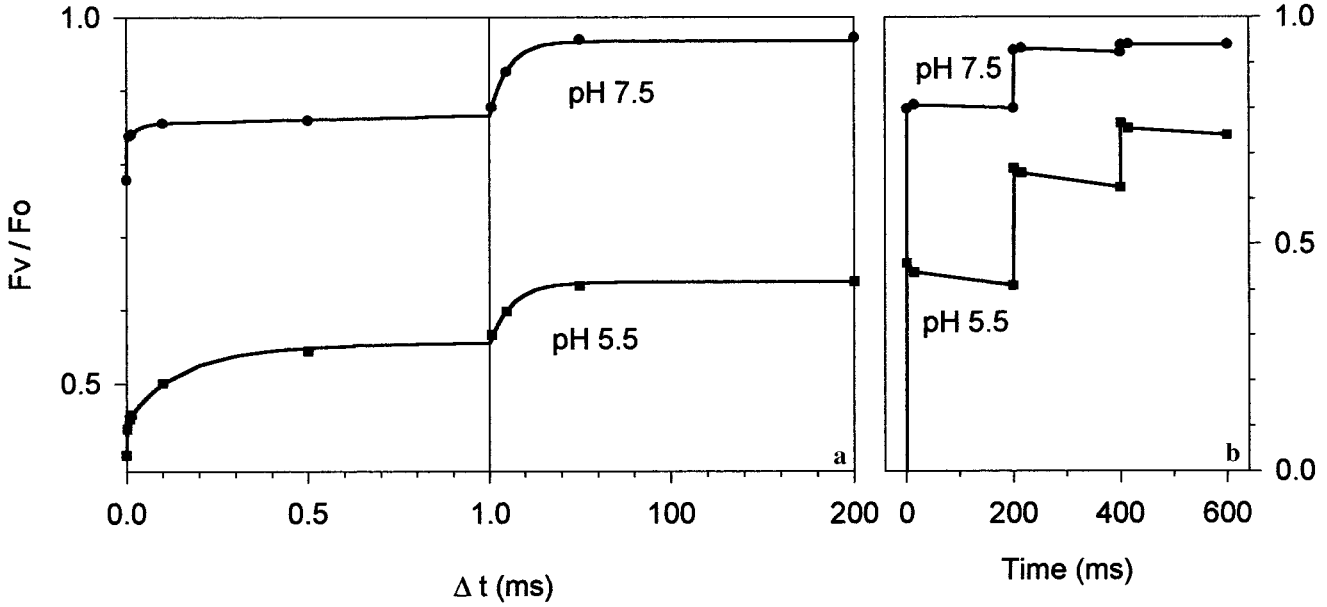

FIGURE 5: Panel a shows a turnover experiment of the fluorescence level in algae inhibited by DCMU (10 μM) and hydroxylamine (2 mM) at pH 5.5 and pH 7.5 (same procedure as in Figure 3). The curves indicate an adjustment with a sum of two exponentials and an unresolved fast phase, with $t_{1/2}$ and relative amplitudes: <5 μs (19%), 98 μs (43%), and 8.5 ms (38%) at pH 5.5; <5 μs (29%), 21 μs (12%), and 7 ms (59%) at pH 7.5. Panel b shows the fluorescence changes induced by the three first flashes of a series on the same material.

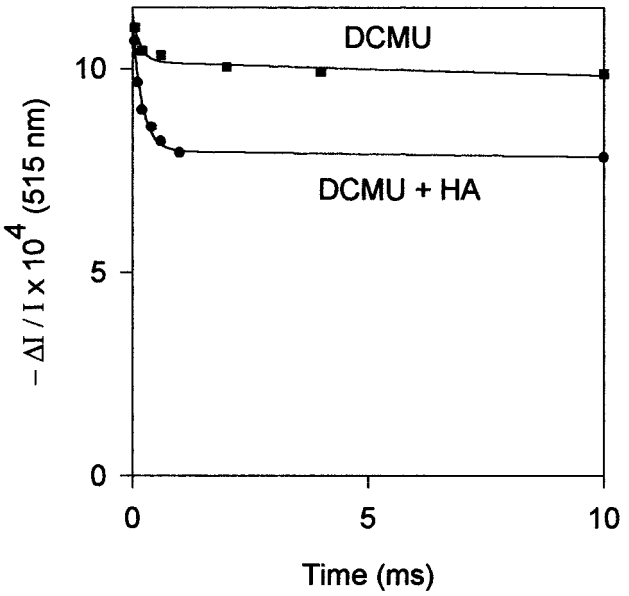

FIGURE 6: Kinetics of the 515-nm absorption changes induced by a xenon flash. Algae at pH 6 in the presence of DCMU, in the absence (squares) or presence (circles) of hydroxylamine. The fast phase was fitted with an exponential decay with $t_{1/2}$ = 145 μs and amplitude = 28% (DCMU + HA).

at 10 μs was found very similar in the absence or presence of hydroxylamine, but in the latter case, a fraction of the signal decays in the 100 μs range. We also monitored the fluorescence yield kinetics in this time domain and observed (not shown) an increase of this signal in the 100 μs range. This is likely indicating a slow phase of P_{680}^+ decay, since this species is known to be an efficient quencher of PS II fluorescence. Taken together, these results suggest that the inefficient yield in the presence of hydroxylamine is due to a fraction of centers where P_{680}^+ reduction by the secondary donor (Y_Z) is slow and which undergo the recombination reaction $P_{680}^+Q_A^- \rightarrow P_{680}Q_A$. We also studied the 515 nm change kinetics in the absence of DCMU and observed (not shown) a similar effect of hydroxylamine, showing that the recombination phase in the 100 μs range also occurs in the absence of acceptor side inhibition (although its extent and rate are somewhat modified due to the shorter lifetime of Q_A^-).

The relative extent of the 100 μs phase of the membrane potential decay after one flash was found dependent on the ambient pH. At pH 5.5, 6, and 7.5, we obtained typical figures of 35%, 30%, and 10%, respectively (implying a charge separation efficiency of 65%, 70%, and 90%, respectively). This is consistent with the fluorescence data, taking into account the hyperbolic dependence of the fluorescence yield on Q_A^-.

Turnover experiments homologous to those using fluorescence were run using the 515 nm change. In this case, one measures the field-indicating change caused by the second flash as a function of the delay (Δt) after the first flash. Panel a in Figure 7 shows the field decay after the first flash (circles), with a recombination phase amounting to about 38% of the initial amplitude (pH 5.5 in this experiment), followed by a slow decay due to ionic permeability across the membrane. The level reached at 50 μs after the second flash is plotted with square symbols, as a function of the Δt between the first and second flashes. For small Δt, this quantity is slightly above the 50 μs level reached after the first flash. It then decays along with the ionic leak, so that the difference between the two curves is constant for Δt > 1 ms, showing that the initial charge separation caused by the second flash is constant after the recombination phase is completed. Therefore, the initial state following the second flash corresponds to 100% charge separation, irrespective of the Δt. A recombination phase in the 100 μs range is also occurring after the second flash, and its amplitude depends on Δt. This is illustrated in the bottom panel, where the relative amplitude of this phase (estimated as the difference between 50 μs and 10 ms) was plotted as a function of Δt. This representation provides an

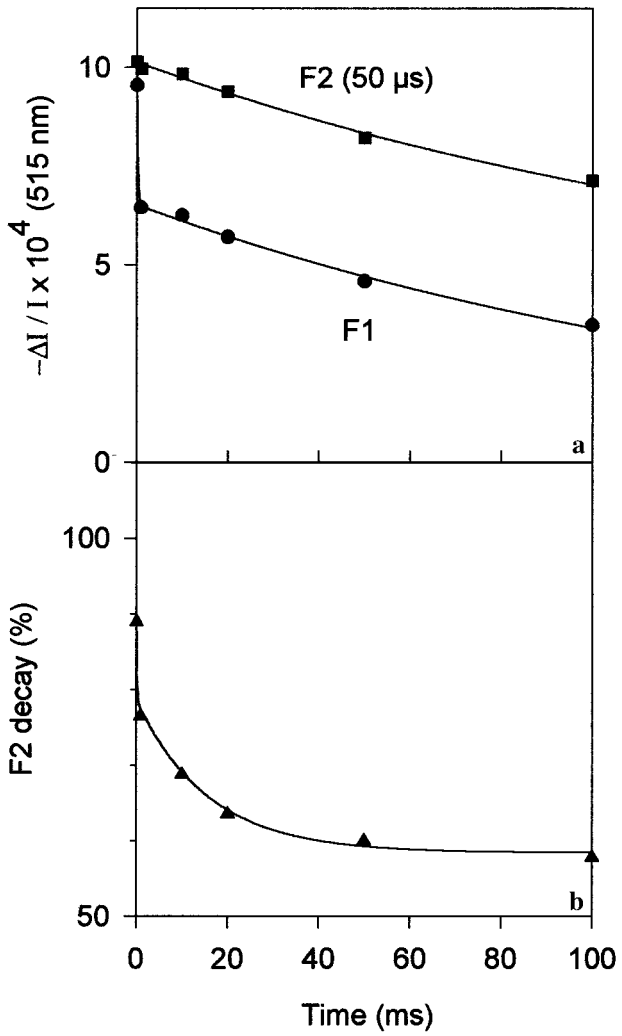

FIGURE 7: 515-nm absorption changes in algae in the presence of DCMU (10 μM) and hydroxylamine (2 mM) at pH 5.5. Panel a: the circles (F1) indicate the kinetics measured after a single ruby laser flash (sampling at 50 μs, 1 ms, 10 ms ...); the squares (F2) indicate the level measured at 50 μs after a xenon flash triggered Δt (horizontal scale) after the laser pulse. The exponential decay curve fitting the F1 curve ($t \geq 1$ ms) was translated by an appropriate offset to fit the F2 data points, showing the constant amplitude of the 515-nm change caused by the second flash. Panel b shows the relative extent of the 100 μs decay following the second flash, as a function of Δt. The difference of the absorption levels sampled at 50 μs and 10 ms was normalized by the offset between the F1 and F2 curves. The curve is a two-exponential fit plus an unresolved fast phase (<10 μs) and an offset, with $t_{1/2}$ and amplitudes: <10 μs (7%), 100 μs (15%), 11 ms (20%), and offset (58%). An additional data point at $\Delta t = 150$ ms (not shown) was taken into account in the fitting procedure. As explained in the text, the time course of the turnover is the complement to 100% of this curve.

inverted plot of the turnover since, starting from 100% charge separation, the extent of the recombination is complementary to that of the charge stabilization at 10 ms. The turnover pattern obtained in this manner is consistent with the fluorescence results of Figure 7, indicating successive phases for the increase in charge stabilization in the <20 μs, 100 μs, and 10 ms domains. These kinetic features were established in a number of experiments where Δt was varied more extensively than in Figure 7. The reason for the scarcity of data points in this experiment was to shorten the overall time of the experiment in order to minimize the effect of drifts in the absorbance change levels. In the experiment of Figure 7 (pH 5.5), the extent of the recombination phase following the first flash is about 38% of the initial amplitude, thus the stabilization yield is 62%. The relative efficiency of the second flash (for $\Delta t \geq 100$ ms) is smaller, about 40%. When similar experiments were run at pH 6.0, a stabilization yield of 70−75% was found for the first flash and 60−70% for the second flash.

DISCUSSION

The occurrence of photochemical misses in the presence of DCMU is revealed by the incomplete charge separation (measured in the millisecond range) induced by one saturating flash. This phenomenon is readily observed by monitoring the fluorescence yield, which is a sensitive indicator of the amount of Q_A^-, especially at high fluorescence yield levels due to the hyperbolic dependence on $[Q_A^-]$. The fact that the fluorescence level reached after a saturating flash is below the maximum F_m level was reported by Doschek and Kok (*30*) and Joliot and Joliot (*23*). The latter authors proposed a model involving two acceptors, Q_1 (equivalent to Q_A) and Q_2, both present in the same PS II center. The photoreduction of Q_1 by a saturating flash was assumed to be essentially 100%, while that of Q_2 would be intrinsically less efficient. The illumination of a dark-adapted sample with a (μs) xenon flash would nevertheless result in substantial reduction of Q_2 because the rapid reduction of P_{680}^+ in the 100 ns range allows double-hitting (i.e., the $P_{680}Q_1^-Q_2 \rightarrow P_{680}^+Q_1^-Q_2^-$ reaction). The lower fluorescence yield reached after one flash in the presence of hydroxylamine was explained on this basis (*23, 31*): because the destruction of the Mn cluster results in a much longer lifetime of P_{680}^+ after the first charge separation, little double-hitting can occur. Consequently, only Q_1 photoreduction would take place on the first flash. A crucial test of this model is obtained by comparing the fluorescence pattern induced by microsecond xenon flashes versus nanosecond laser flashes. If the effect of hydroxylamine is due to the suppression of double hits, this should be mimicked, in the absence of hydroxylamine, by using a nanosecond flash that has a short duration with respect to the P_{680}^+ lifetime in the native oxygen-evolving system. As shown in Figure 2, the fluorescence pattern is in fact very similar with xenon or laser flashes, in disagreement with the dual acceptor model. A similar conclusion was reached by Döring et al. (*32*)and Eckert et al. (*33*) from a study of P_{680}^+ absorption changes.

The present results are consistent with an interpretation of the photochemical misses observed in the presence of DCMU as caused by a recombination reaction competing with charge stabilization on the donor side. The enhancement of the miss probability induced by hydroxylamine is a consequence of the slower donor side reactions. The pH dependence of this effect is also (qualitatively, see below) in line with the slower P_{680}^+ reduction rate observed at low pH in the Mn-depleted system. Supporting the occurrence of a transient recombination, we observed a decrease of Q_A^- (C-550) in the 100 μs range, accompanied with a decay of the membrane potential monitored through the carotenoid absorption shift and with a rising phase of the fluorescence yield indicating that a fraction of P_{680}^+ is decaying in this time range. The C-550 or 515 nm changes sampled at a short time after the flash indicate that the initial extent of

charge separation is similar irrespective of the presence of hydroxylamine, so that the lower overall yield caused by hydroxylamine appears essentially due to the increased recombination.

The turnover experiments, measuring the efficiency of a second flash as a function of the delay after the first flash, revealed multiphasic kinetics involving three distinct time domains, roughly <10 μs, 100 μs, and 10 ms. As discussed above, the middle phase (100 μs) is concomitant with a recombination process. This is also most probably the case for the faster phase (<10 μs). Conjeaud and Mathis (*26*) investigated the reduction kinetics of P_{680}^+ and their pH dependence in Tris-washed thylakoids (a treatment that, like hydroxylamine, causes inactivation of the oxygen-evolving complex by destruction of the Mn cluster). The kinetics were analyzed as a sum of two exponential components: a fast phase with a markedly pH-dependent rate and a slow phase with $t_{1/2} \approx 140$ μs and no significant pH dependence. The relative amplitude of the fast phase was 75−90%, with a weak pH dependence (decreasing trend toward high pH). At pH 5.5, the $t_{1/2}$ of the fast phase was about 10 μs, 7 μs at pH 6, and 3.5 μs at pH 7.5. The $t_{1/2}$ of the back-reaction between P_{680}^+ and Q_A^- was found about 130 μs, irrespective of pH. It may be seen that these figures do not quite match the large flash inefficiency observed in the presence of hydroxylamine and DCMU at low pH. For instance, at pH 5.5, we found about 40% misses, whereas the data of Conjeaud and Mathis predict less than 22% (i.e., taking the maximum extent of the slow phase and assuming it is entirely due to recombination, its contribution would be 15%, and an additional 7% would come from the stabilization−recombination competition in the fast-decaying centers). Our data rather suggest a larger fraction of the 140 μs decay phase at low pH. This discrepancy may be due to the use of 5 mM ferricyanide in Conjeaud's experiments or to the use of DCMU in ours. Notably, ferricyanide is expected to oxidize the non-heme iron in the vicinity of the quinone acceptors, which then becomes an efficient competitor for the reoxidation of Q_A^- (*34*), thus diminishing the extent of the recombination. Some competition with the normal Q_A^- to Q_B transfer is also expected to occur in the absence of DCMU.

We come now to the unexpected and most interesting feature in the turnover results, namely, the phase occurring in the tens of milliseconds domain. In this time range, no decay of Q_A^- (or P_{680}^+) is observed. Hence, the process that underlies this recovery phase cannot be ascribed to a reopening of closed centers but must be due to a change concerning open centers. What happens during this process is enlightened by the behavior of the field-indicating kinetics induced by the second flash. The initial change caused by this flash is constant in the 1−200 ms range of the time delay after the first flash, indicating that all open centers are photochemically competent. The increase in the extent of the final charge separation corresponds to a decrease in the amplitude of the recombination phase occurring in the 100 μs range. This observation leads to the following model. We assume a conformational equilibrium in PS II, between two states differing by their efficiency in the recombination−stabilization competition. In one conformation, the centers are good stabilizers, and bad stabilizers in the other conformation. The efficient and unefficient conformations are likely to correspond to the populations where P_{680}^+ reduction is, respectively, rapid (5−10 μs range in Mn-depleted PS II) and slow (100 μs range). The centers that become permanently closed on the first flash are predominantly the efficient ones, so that the second flash addresses predominantly the unefficient centers. The process that takes place in the 10 ms time range reflects the relaxation of the conformation equilibrium, during which the "photoselected" unefficient centers redistribute between the efficient/unefficient states. This equilibrium model implies that the two populations are interconvertible rather than fixed, heterogeneous states. The fast phase of the turnover kinetics corresponds to the small fraction of efficient centers which recombined during the 5−10 μs lifetime of their P_{680}^+ (here and in the following, we comment the data obtained for pH 5.5−6). This fraction will be efficiently closed by the second flash for $\Delta t > 10$ μs. To account for the second phase of the turnover, accompanying the 100 μs decay phase of P_{680}^+, one must assume that the inefficient centers are not totally so. The centers that have just reopened during this phase do give an additional fraction of charge stabilization on the second flash, which indicates that their efficiency is not zero but about 10−20%. Finally, an equilibrium relaxation takes place, tending to restore partially the initial distribution of efficient and inefficient centers (10 ms phase of the turnover). This restoration is not complete since the relative efficiency of the second flash (for $\Delta t \geq 100$ ms) was consistently found smaller (especially at low pH) than that of the first flash. This raises the possibility of a very slow phase of the relaxation process. We observed, however, a constant efficiency of the second flash when increasing Δt from 100 ms to 1 s. Therefore, if a slow relaxation phase exists, it must occur in a time domain $\gg 1$ s.

A detailed account of the effects of pH on the various parameters involved in our model is beyond the present scope. It is however clear that lowering the pH increases the relative amount of inefficient centers and also the fraction of centers that remain in an inefficient conformation on a long time scale (> 1 s). On the other hand, the rate of the 10 ms relaxation phase seems little affected by the pH.

Because of the smaller extent of the photochemical misses in the absence of hydroxylamine (with DCMU present), we could only investigate the turnover process through fluorescence experiments. The qualitative similarity of these results with those obtained in the presence of hydroxylamine is striking and suggests that the model proposed above remains essentially valid when hydroxylamine is absent. In particular, the occurrence of a 10 ms turnover phase that cannot be associated with any electron transfer process provides evidence for a similar dynamic heterogeneity, controlled by a conformation equilibrium. The phases in the microsecond and tens of microsecond ranges are probably due to partial recombination in centers responsible for the slower phases of P_{680}^+ reduction that were observed by a number of authors (*11*, *35*, *36*). Some of these phases (35 μs or faster) display an S-dependent amplitude, whereas this does not seem to be the case for slower phases that may or may not be associated with centers with a nonfunctional water oxidase. It has been recently proposed (*36*) that the 35 μs phase reflects a stabilization of Y_Z^+ (i.e., a progressive increase of the equilibrium constant between Y_Z^+ and P_{680}). The present results do not exclude this possibility but suggest that the kinetic heterogeneity of P_{680}^+ reduction is, at least in part,

linked to the conformational equilibrium between bad and good stabilizing centers. It cannot be decided at the present stage which of the slow phases (35 μs or longer) are associated with the inefficient conformation.

The analysis of the fluorescence pattern observed in DCMU-inhibited material submitted to a series of saturating flashes suggests that, especially at low pH, an additional heterogeneity must be taken into account. The very slow rise beyond the second flash indicates a small fraction of centers (2−5%) with very low flash yield (30−50%). These estimates are derived from the data of Figure 1, together with unpublished results (see also refs *30* and *23*). This phenomenon also appears as a slow phase in fluorescence induction curves, denoted "γ" by Hsu et al. (*37*, *38*), and may interfere with analysis of the α and β contributions (see the review; ref *39*). This feature is qualitatively similar to the observation discussed earlier that, in the presence of hydroxylamine, a fraction of centers remains blocked in an inefficient state either permanently or on a long time scale.

The existence of manifolds of conformational states in proteins is broadly accepted (*40*). Modulation of reaction centers kinetics by conformation equilibria has been reported in several studies (*41*−*43*). The evidence reported in the present paper suggests that the multiphasic kinetics of P_{680}^+ reduction, observed in native or Mn-depleted PS II, is controlled by such a process with a relaxation time of 10−20 ms. This phenomenon together with the electron transfer equilibrium between the quinone acceptors is responsible for the randomly distributed photochemical misses observed in PS II.

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