Misses during Water Oxidation in Photosystem II Are S State-dependent*

Guangye Han, Fikret Mamedov, and Stenbjörn Styring

From the Chemistry-Ångström, Box 523, Uppsala University, 751 20, Uppsala, Sweden and the Department of Chemistry, Umeå University, Linnaeus väg 6, 901 87 Umeå, Sweden

Background: Misses in Photosystem II (PSII) hinder the advancement of the S cycle.

Results: Misses were directly measured by the EPR spectroscopy and found to be different for different S transitions.

Conclusion: Fraction of the miss originates in the reactions at the CaMn₄O₅ cluster.

Significance: Implications for understanding the water oxidation mechanism in PSII are revealed.

The period of four oscillation of the S state intermediates of the water oxidizing complex in Photosystem II (PSII) is commonly analyzed by the Kok parameters. The important miss factor determines the efficiency for each S transition. Commonly, an equal miss factor has been used in the analysis. We have used EPR signals which probe all S states in the same sample during S cycle advancement. This allows, for the first time, to measure directly the miss parameter for each S state transition. Experiments were performed in PSII membrane preparations from spinach in the presence of electron acceptor at 1 °C and 20 °C. The data show that the miss parameter is different in different transitions and shows different temperature dependence. We found no misses at 1 °C and 10% misses at 20 °C during the S₁→S₂ transition. The highest miss factor was found in the S₂→S₃ transition which decreased from 23% to 16% with increasing temperature. For the S₃→S₀ transition the miss parameter was found to be 7% at 1 °C and decreased to 3% at 20 °C. For the S₀→S₁ transition the miss parameter was found to be approximately 10% at both temperatures. The contribution from the acceptor side in the form of recombination reactions as well as from the donor side of PSII to the uneven misses is discussed. It is suggested that the different transition efficiency in each S transition partly reflects the chemistry at the CaMn₄O₅ cluster. That consequently contributes to the uneven misses during S cycle turnover in PSII.

Photosystem II (PSII), in plants, algae, and cyanobacteria carries out the light-induced oxidation of water (1–3). After initial charge separation between P₆₈₀ and pheophytine, the electron is transferred via the two quinone acceptors, Qₐ and Qₜ to the plastoquinone pool to be utilized in subsequent reactions in the thylakoid membrane. P₆₈₀⁺ is a highly oxidising species and extracts electron from the nearby redox-active tyrosine residue, Y₁, together with the CaMn₄O₅ cluster compose the water-oxidizing complex, the catalytic site where oxidation of water occurs (4–6). The crystal structure of PSII from thermophilic cyanobacteria has been available for a few years (7–9), and recently, the organization and coordination of the CaMn₄O₅ cluster became available at 1.9-Å resolution (10).

Four consecutive charge separations in PSII are necessary to oxidize two water molecules to molecular oxygen. During this process, four protons are released from the water-oxidizing complex (WOC). In this enzymatic turnover, the WOC cycles through five intermediate redox states, collectively called the S states, labeled S₀→S₄ (Fig. 1) (11, 12). S₀ is the most reduced state whereas S₁, S₂, and S₃ represent sequentially higher oxidation states in the WOC. S₅ is the dominating state in the dark. S₂ and S₃ states are metastable and decay back to the S₁ state in a few minutes at room temperature (13, 14) whereas the S₀ state is slowly (in tens of minutes) oxidized to the S₁ state by Y₁⁺, the second redox-active tyrosine in PSII (15). O₂ is released during the S₁→[S₄]→S₀ transition, where S₄ is a transient state (Fig. 1) (4–6).

The S cycle model was introduced by Kok and co-workers (11, 12) in studies of the flash-dependent oxygen release when a dark-adapted suspension of Chlorella cells was exposed to a train of light flashes (16). The oxygen yield was maximal on the third flash and oscillated with period of four thereafter. The oscillation pattern dampens with the number of flashes, eventually leading to constant oxygen yield on each flash.

Both the oscillation and the dampening were explained in the S cycle model, and the concepts of double hits, backward transitions (recombination reactions), and misses were introduced (Fig. 1). In the model, double hits (β) reflect a double turnover of the WOC as a result of one flash (11, 12), and they are frequent when flashes from xenon flash lamps (tens of microseconds long) are used.

The miss parameter (α) is the most important factor in understanding the S cycle advancement and dampening of the oscillations in the oxygen release. A miss represents a probability of the WOC to fail in advancing in the S state cycle when the PSII center is exposed to a flash. The terminology “miss” is...
In the last decade we have developed S state-specific EPR probes to the \( S_1 \), \( S_3 \), and \( S_0 \) states (36, 37). These EPR signals, called Split EPR signals, originate from the \( Y_z \) radical in magnetic interaction with the \( \text{CaMn}_4\text{O}_5 \) cluster in the corresponding S state (37, 38). In combination with the well known \( S_0 \) state multilane EPR signal, these Split EPR signals provide a direct possibility to quantify all S states independently, even in the very same sample. In a previous study we have demonstrated this method in PSII samples predominantly placed in different S states after application of partially saturating laser flashes (36). Thereby, we now possess spectroscopic probes to quantify every S state of the S cycle of the WOC and moreover, to deconvolute the S state distribution in the same sample.

In this study, by using this approach we directly measure the distribution of all S states in the same sample after the application of every turnover flash. This allows us to determine the miss parameter for each individual S state transition during the catalytic turnover of PSII. We show that the miss parameter is very different in the different S state transitions.

**EXPERIMENTAL PROCEDURES**

**Preparation of PSII and EPR Samples**—PSII membranes (39, 40) were diluted to 2 mg of Chl/ml in 25 mM Mes-NaOH (pH 6.3), 400 mM sucrose, 5 mM MgCl_2, 10 mM NaCl buffer, and filled into calibrated EPR tubes. The EPR samples were exposed to room light at 20 °C for 5 min to fully oxidize \( Y_D \), and were then dark-incubated for 15 min. Thereafter, PSII was synchronized in the \( S_0 \) state by the application of two saturating pre-flashes from a \( \text{Nd:YAG} \) laser from Spectra Physics (6 ns, 840 mJ, 532 nm, 1.25 Hz) followed by a dark incubation for 30 min at 20 °C (35, 36). This procedure results in formation of the \( S_1Y_D^\ast \) state in 100% of PSII centers. PpBQ was added to a final concentration of 0.5 mM. 30 s after the addition of PpBQ the samples were transferred to an ethanol bath at 1 °C and 20 °C. After temperature equilibration for 3 min, the samples were exposed to saturating turnover flashes (from 0 to 6) at the frequency of 1.25 Hz (800 ms between the flashes) and frozen within 1 s in an ethanol-dry ice bath at 198 K and then transferred to \( N_2 \).

**EPR Spectroscopy**—EPR measurements were performed with a Bruker ELEXYS E500 spectrometer with a SuperX ER 049X microwave bridge and a ER 412SHQE-LC cavity. The spectrometer was equipped with an ESR 900 liquid helium cryostat and ITC 503 temperature controller from Oxford Instruments (Oxfordshire, UK). Illumination into the EPR cavity at 5 K with visible light (160 W/m², white light lamp projector, 4 min) to induce the Split \( S_1 \) and \( S_0 \) EPR signals and 830-nm light (280 W/m², LQC830-135E laser diode, Newport, RI, 10 min) to induce the Split \( S_3 \) EPR signal were carried out as in Ref. 36. The fractions of centers in the \( S_1 \), \( S_2 \), \( S_3 \), and \( S_0 \) states after different number of flashes were determined in the same sample from the intensities of the Split \( S_1 \), \( S_3 \) multilane, Split \( S_0 \), and Split \( S_0 \) EPR signals (Fig. 2), respectively, as described earlier (36). Each flash series experiment at a defined temperature was carried out at least two times with a difference between the determined S state distribution in a certain sample of <4% (S.E.).
The $S_1$ state is stable in the dark and is present in 100% of the PSII centers after application of the preflashing procedure (15, 28, 35, 36). The $S_2$, $S_3$, and $S_0$ states dominate in the samples after the application of one, two, or three saturating laser flashes, respectively. Freezing of the flashed PSII samples within 1–2 s after the last flash retains the redox configuration of the WOC in the corresponding S state and allows further spectroscopic investigations.

Fig. 2 shows the EPR signals that we have used to access these $S$ states. The Split $S_1$ (A), Split $S_3$ (C), and Split $S_0$ (D) signals were induced by illumination at 5 K and were used to analyze the $S_1$, $S_3$, and $S_0$ state, respectively. The $S_2$ state multiline signal, formed directly after one flash (Fig. 2B) was used to analyze the $S_2$ state. Taken together, these signals allowed us to have a complete picture of the $S$ state distribution in PSII in each sample.

Quantification of $S$ State Distribution after Application of Turnover Flashes

1 °C—The synchronized PSII samples were given zero to six saturating flashes at a frequency of 1.25 Hz. Fig. 3 shows EPR spectra recorded in samples flashed at 1 °C. The $S$ state distribution was determined by EPR spectroscopy and is shown in Table 1. In our dark-adapted samples (zero flashes, spectra a) we observed only the Split $S_1$ EPR spectrum (Fig. 3A, spectrum a). This represents 100% of the PSII centers (36). There was no lingering (after the preflash) $S_0$ state in this sample as evidenced by the lack of the $S_2$ state multiline signal (Fig. 3B, spectrum a). After one flash (spectra b), the $S_2$ state multiline signal was formed (Fig. 3B, spectrum b) whereas the Split $S_1$ signal had completely disappeared (Fig. 3A, spectrum a). This indicates 100% efficiency in the $S_1$ to $S_2$ state turnover at 1 °C and our flashing conditions (Tables 1 and 2) as was reported by us earlier (36). In the same publication the very small underlying signal, visible in Fig. 3A, spectrum b, was shown not to originate from the $S_1$ state (36).

After two turnover flashes the $S_3$ state was formed in the majority of the centers. 23% of the PSII centers had not advanced to the $S_2$ state (Table 1) as could be determined from the $S_2$ state multiline signal that remained after the two flashes (Fig. 3B, spectrum c). The remaining 77% of PSII advanced to the $S_3$ state. These centers gave rise to a large Split $S_3$ EPR signal (Fig. 3, A and C, spectra c) under both visible and NIR illumination at 5 K. Thus, a miss involving 23% of PSII was observed in the $S_2\rightarrow S_3$ transition at 1 °C (Table 2).

Application of three flashes (Fig. 3, spectra d) resulted in samples where the $S_0$ state was dominating (72%, Table 1). 5% of PSII remained in the $S_2$ state (Fig. 3B, spectrum d, small $S_2$ multiline contribution) and 23% in the $S_3$ state (Fig. 3C, spectrum d showing a quite large Split $S_3$ signal; Table 1). The $S_0$ state was correlated to the induction of the Split $S_1$ EPR signal (Fig. 3A, spectrum d) as described earlier (36). Thus, in the $S_0\rightarrow S_1$ transition, during the oxygen formation and release step, a miss factor of 7% was observed at 1 °C (Table 2).

In a similar way we dissected the $S_0\rightarrow S_1$ state transition in samples subjected to four flashes at 1 °C. The EPR spectra are presented in Fig. 3 (spectra e). Illumination of the samples with visible light resulted in formation of predominantly the Split $S_1$ EPR signal with some contribution of the Split $S_0$ signal (Fig. 3A, spectrum e). Traces of the Split $S_2$ EPR signal were also observed whereas the $S_2$ multiline signal was absent. Complete disappearance of the $S_2$ multiline EPR signal after three or four flashes (Fig. 3B, Table 1) is indicative of absence of a population of PSII centers that is “stuck” in the $S_2$ state in our preparations (30, 31). The Split $S_3$ signal was estimated to correspond to 7% of the total amount of PSII centers (Fig. 3, A and C, spectra e). 23% of the centers still remained in the $S_0$ state whereas the majority of the centers (65%) had advanced to the $S_1$ state. This is represented by the dotted spectrum e in Fig. 3A of the Split $S_1$ signal obtained after subtraction of the corresponding fractions of the Split $S_3$ and Split $S_0$ signals (36). From this, the miss factor in the $S_0\rightarrow S_1$ transition was calculated to be 10% at 1 °C (Table 2).

Application of five or six flashes advanced our PSII samples to, dominantly, the $S_2$ and $S_3$ states of the second turnover of
PSII, respectively. This is illustrated by the reappearance of the S₂ state multiline (Fig. 3B, spectrum f) and Split S₃ EPR signals (Fig. 3, A and C, spectra g). Analysis of these spectra revealed the S state distribution in the PSII samples after application of five and six flashes (Table 1). The miss factors of the second turnover S₁ → S₂ and S₂ → S₃ state transitions were found to be

**TABLE 1**

Distribution of the different S states (percentage of PSII total) in PSII samples after the application of zero to six turnover flashes given at 1.25-Hz frequency at 1 °C and 20 °C

| Temperature and flash no. | S₁ | S₂ | S₃ | S₀ | S₁(2nd) | S₂(2nd) | S₃(2nd) | Total |
|---------------------------|----|----|----|----|---------|---------|---------|-------|
| 1 °C                      |    |    |    |    |         |         |         |       |
| 0                         | 100| 100| 100| 100|         |         |         |       |
| 1                         | 100| 100| 100| 100|         |         |         |       |
| 2                         | 23 ± 1| 77 | 72 | 65 |         |         |         | 97    |
| 3                         | 5 | 22 ± 1| 23 | 66 |         |         |         | 101   |
| 4                         | 3 | 10 ± 1| 22 | 66 |         |         |         | 101   |
| 5                         | 3 ± 1| 8 | 35 | 53 |         |         |         | 99    |
| 6                         | 6 | 9 | 37 ± 2| 52 |         |         |         |       |
| 20 °C                     |    |    |    |    |         |         |         |       |
| 0                         | 100| 100| 100| 100|         |         |         |       |
| 1                         | 23 ± 1| 76 | 74 | 66 |         |         |         | 99    |
| 2                         | 5 | 22 ± 1| 22 | 66 |         |         |         | 101   |
| 3                         | 4 | 22 ± 3| 9 | 24 |         |         |         | 97    |
| 4                         | 3 | 9 | 37 ± 2| 52 |         |         |         |       |
| 5                         | 4 | 9 | 37 ± 2| 52 |         |         |         |       |
| 6                         | 6 | 9 | 37 ± 2| 52 |         |         |         |       |
almost identical to the corresponding miss factors in the first
turnover of the WOC (Table 2).

20 °C—It is known that the S state transitions strongly
depend on the temperature (18, 23, 41). The experiments
described above were performed at 1 °C. We have performed a
similar set of experiments also at 20 °C. The results are shown in
Tables 1 and 2. The exact value of the miss factor (α) was deter-
mainted the same way as described above, directly from the frac-
tion of PSII centers that did not advance to the next S state after
the turnover flash.

We observed no misses in the $S_1 \rightarrow S_2$ transition at 1 °C. At
20 °C, this transition was not as effective. 10% of the PSII cen-
ters did not advance to the $S_2$ state at 20 °C (Tables 1 and 2).
The $S_2 \rightarrow S_3$ transition was different. The highest miss factor
was found for this transition also at 20 °C. At 1 °C the miss
factor was determined to be 23%. The temperature dependence
was opposite if compared with the $S_1 \rightarrow S_2$ transition, and
the efficiency of transition increased with increased temperature
(decreased to 16% misses at 20 °C; Tables 1 and 2).

The efficiency of the $S_3 \rightarrow S_0$ and the $S_0 \rightarrow S_1$ transitions was
also somewhat temperature-dependent (Tables 1 and 2). The miss
factors for these transitions were correspondingly 7 and
10% at 1 °C and stayed almost the same (11%) with increasing
temperature for the $S_0 \rightarrow S_1$ transition and decreased to 3% for
the $S_3 \rightarrow S_0$ transition at 20 °C (Tables 1 and 2).

### Involvement of Side Path Donors

In the $S_2 \rightarrow S_3$ transition we found a large miss at both
temperatures. It is possible that such a large miss could be accom-
panied by measurable oxidation of side path donors instead of
the WOC. We therefore investigated the oxidation of Chl$_z$ and
Cyt $b_{559}$ after one and two flashes at 1 °C by EPR measurements.
In neither case was Chl$_z$ or Cyt $b_{559}$ oxidized to any detectable
extent (data not shown). This indicates that the large misses in
WOC turnover did not involve competition from the carote-
noid/Chl-Cyt $b_{559}$ pathway.

### DISCUSSION

**Significance of Kok Parameters in Our Analysis: Involvement
of Recombination Reactions**—The discovery of the flash-depen-
dent oscillation of the oxygen evolution by PSII and the intro-
duction of the S cycle concept were a quantum leap in understand-
ing of photosynthetic water splitting (11, 12, 16). The dampening
of the oscillation with increasing flash number was
phenomenologically explained with misses, backward transi-
tions (recombination reactions), and double hits (Fig. 1). These
concepts are routinely used in the analysis of the S cycle, mostly
in flash-induced oxygen evolution studies where it was intro-
duced (19, 20, 22–27). They have also been used in the analysis
of S cycle intermediates studied with EPR spectroscopy (28, 35,
36), transient optical spectroscopy, variable fluorescence (21,
41–43), and even EXAFS (30, 31, 33, 34), reflecting S state-de-
pendent structural changes.

Double hits normally originate from double turnover in the S
cycle during a long xenon flash. Such flashes were used earlier.
In more recent experiments and in our work here much shorter
nanosecond laser flashes are used (36). This is an important
achievement that simplifies our analysis because our EPR mea-
surements directly demonstrate the absence of double hits in
our experiments. We did not observe any EPR signals from the
$S_3$ state after one flash, from the $S_2$ state after two flashes, etc.
(Fig. 3). Thus, our flashing protocol eliminates double hits from
the experiments. Consequently, we conclude that they are not
an inherent property in PSII and WOC function. Their exist-
ence is an artifact that reflects the application of too long
flashes in the experiment.

Oxidation of $Y_{1D}$, which sometimes present in the PSII
samples, by the $S_2$ or $S_3$ state can occur in a few seconds time
range. If it occurred this would also result in a miss (24, 26).
However, with EPR, the oxidation level of $Y_{1D}$ is easily controlled
by a direct measurement. Our well established prefloashing pro-
duct results in 100% oxidized $Y_{1D}$ before the turnover flashes
(28, 35, 36). This was also verified in every individual sample
used here. In particular, we emphasize that the amplitude of $Y_{1D}$
was maximal (100%) in the sample that was frozen immediately
after the addition of PpBQ (the zero flash sample). Therefore,
we can exclude any contribution from $Y_{1D}$ in the miss forma-
tion in our experiments.

Misses due to recombination reactions from the quinone
acceptor system have an internal origin and are the property
of electron transfer equilibria in PSII. Recombination reactions
can involve either the $S_2$ or $S_3$ states of the WOC. They can also
involve P$_{680}^+$ or Y$\cdot$ if for some reason the WOC is slow or
nonfunctional. The first case reflects $S_0$ or $S_1$ state recom-
bination with QA$^-$. Here, the WOC has in fact turned over, but
recombination with a lingering electron on the acceptor side
leads to a backward transition in the S cycle (Fig. 1, dash-dotted
arrow). The second case reflects P$_{680}^+$ or Y$\cdot$ recombination
with QA$^-$. In this situation the WOC never had the opportunity
to turn over. The effect on for example flash-induced oxygen
release is the same, but the underlying mechanism is totally
different.

Our experiments have been done in presence of sufficient
concentrations of the electron acceptor, PpBQ, to ensure the
efficient forward electron transfer from QA$^-$ after each flash.
Recently, we reported the decay kinetics of the $S_2$ and the $S_3$
states in the presence of PpBQ at similar experimental condi-

### TABLE 2

| Temperature | $S_1 \rightarrow S_2$ | $S_2 \rightarrow S_3$ | $S_0 \rightarrow S_3$ | $S_0 \rightarrow S_1$ | $S_1 \rightarrow S_2$ (2nd) | $S_2 \rightarrow S_3$ (2nd) | Total$^b$ | Average$^c$ |
|-------------|------------------|------------------|------------------|------------------|------------------|------------------|---------|---------|
| 1 °C        | 0                | 23               | 7                | 3                | 0                | 20               | 40      | 10      |
| 20 °C       | 10               | 16               | 3                | 11               | 8                | 15               | 40      | 10      |

$^a$ Miss parameters of the transitions of the second turnover of S cycle.
$^b$ Sum of misses for all S transitions of the first turnover of the S cycle.
$^c$ Average miss for every transition of the first turnover of the S cycle.
tions. They are biphasic, and in the range of tens of seconds for the fast phase which involved only 10–15% of the \(S_2\) or \(S_3\) state (14). We have used 800-ms spacing time between the flashes, and our freezing of the EPR sample after the flash occurs within 1–2 s. Both are fast compared with the fast \(S\) state decay. Therefore, we can safely discard recombination reactions from \(Q_A^-\) involving the CaMn\(_4\)O\(_5\) cluster and any connected decay of the \(S_2\) and the \(S_3\) states between the flashes from our analysis at 1 °C (14). At 20 °C we can however not exclude recombination involving the \(S_2\) state in a small fraction of PSII centers (<5%) before the sample was frozen (14).

However, recombination reactions between \(Q_A^-\) and \(P_{680}^+\), or even \(Y_Z\) in some cases, can significantly contribute to the misses observed during the \(S\) state transitions (17, 18, 21, 44). These recombination reactions occur in a similar time range as the \(S\) transitions. Recombination between a lingering electron on \(Q_A^-\) and \(P_{680}^+\) is multiphasic with the dominating decay phase of about 200 \(\mu\)s (45). Recombination between \(Q_A^-\) and \(Y_Z\) is slower, in the range of tens of milliseconds in PSII without the CaMn\(_4\)O\(_5\) cluster (46). In both cases, these recombination rates are much faster than our 1–2-s freezing time of EPR samples after application of turnover flashes. Because our EPR measurements are restricted to the \(S\) state intermediates it is not possible to distinguish whether a miss caused by these recombination reactions has involved \(P_{680}^+\) or \(Y_Z\) as a partner on the donor side.

Moreover, these recombination reactions occur in the same time range as the electron transfer from \(Q_A^-\) to PpBQ (microseconds to milliseconds time range, similar to the reactions in the native system as was estimated from the flash-induced fluorescence decay measurements; data not shown). Therefore, we cannot completely rule out the contribution from recombination between \(Q_A^-\) and \(P_{680}^+\) or \(Y_Z\) in the miss formation in our experiments. Although it is difficult to estimate the degree of this contribution it is more likely to be higher at 1 °C. It is probably also flash number-independent.

**Miss Parameters Vary with \(S\) States**—It has sometimes been discussed that the miss factor could be \(S\) state-dependent (11, 17, 21, 27, 47). \(S\) state-dependent misses were predicted from known equilibrium constants on the donor and acceptor sides of PSII (17, 20) and, more recently, estimated from analysis of the flash-induced variable fluorescence yield (21). Despite this, an \(S\) state-independent miss parameter is used predominantly during analysis of the \(S\) cycle intermediates (19, 22–25, 27). There are two reasons for this (20). The first is experimental, and it was found that an equal miss parameter in each \(S\) transition can provide a satisfactory fit to the data when only one parameter is measured (20, 24) (also see the average miss parameter in Table 2). The second reflects the fact that finding the \(S\) state dependence of the miss factor and the individual \(S\) state composition after a flash from the oxygen-evolving step alone is an underdetermined problem (25, 27). It does not allow separate and direct determination of the misses in each \(S\) transition. This argument holds for all measurements that monitor only one \(S\) state, for example also for the oscillation of the \(S_2\) state multiline EPR signal (28–31, 33–35).

We have overcome this problem. Our experimental protocol (36) allows us to quantify the complete \(S\) state distribution directly in a given PSII sample after the application of flashes. Thus, we can, for the first time, measure the miss parameter for all \(S\) state transitions directly (Tables 1 and 2, Fig. 4).

A clear result in our study is that the \(S_1\)→\(S_2\) transition proceeds with a 100% efficiency at low temperatures. Already this result indicates that the miss factor varies with the \(S\) transitions as the average miss at for example 1 °C in our experiment here (10%, Table 2) fits well with other data (17–28). However, if there is no miss in the first flash, there must be more misses in subsequent flashes. This result also shows that our experimental conditions (laser flash power, Chl concentration, etc.) allow charge separation and complete turnover in all PSII centers after a flash.

The highest miss factor was found for the \(S_2\)→\(S_3\) transition; our directly measured value correlates with estimated miss values for this transition (20, 26). The \(S_1\)→\(S_2\) and \(S_2\)→\(S_3\) transitions were also measured for the second turnover (Tables 1 and 2), and at both temperatures the miss factor was found to be the same as for the first turnover.

This result is in contradiction with an earlier study which reported a miss of 5–10% in the \(S_2\) state (21). The largest miss was found for the \(S_2\)→\(S_0\) transition (21). This discrepancy could be related to the different experimental conditions. The results in (21) were obtained by an indirect method in the absence of an added electron acceptor. Therefore, the presence of recombination reactions in a large fraction of the PSII centers most probably influenced the determination of the miss parameters.

In our case, the misses in the \(S_2\)→\(S_0\) and \(S_0\)→\(S_1\) transitions were found to be only about 10% at 1 °C. At 20 °C the miss parameter for the \(S_2\)→\(S_0\) transition was lower (3%) than for the \(S_3\)→\(S_1\) transition (11%, Table 2, Fig. 4). They were always more efficient than the \(S_2\)→\(S_3\) transition. This is not entirely surprising because recent studies have shown that O₂ release during the \(S_2\)→\(S_0\) transition is not limited thermodynamically and highly exothermic, indicating that possibly this is not the most difficult transition in the \(S\) cycle (48, 49).

**Origin of Uneven Misses during \(S\) Cycle: Involvement of Donor Side Reactions**—Our data clearly show that each transition in the \(S\) cycle occurs with a different efficiency. Above we have
Misses and S Transitions in Photosystem II

discussed the contribution from the recombination reactions in the miss parameter. In the presence of PpBQ the acceptor side efficiency to accept electrons should be the same in all S state transitions. Therefore, it is more likely that the differences in the miss parameter between the S transitions originate in the catalytic events at the WOC. We propose that this specific fraction of the misses, connected to the efficiency of the WOC in the particular S state is designated as the “transition efficiency.”

If we analyze the transition efficiency with respect to reactions at the CaMn$_4$O$_5$ cluster, much insight can be gained already from a comparison of the $S_1 \rightarrow S_2$ transition with all of the other S transitions. At lower temperatures $S_1 \rightarrow S_2$ proceeds without any miss, the transition efficiency is 100%. This is unique among the reactions in the S cycle. The chemistry in $S_1 \rightarrow S_2$ is unique in several other aspects too. It is the only transition that is pH-independent (between pH ~4 and 8.5) (35, 50), and it is known to be the only step that involves only electron transfer and no proton release from the WOC (5, 51). The $S_1 \rightarrow S_2$ transition is also operational at much lower temperatures than all of the other steps (13). This probably reflects that the structure of the CaMn$_4$O$_5$ cluster is unaltered in this step as shown by EXAFS spectroscopy (31, 52–54).

The situation is very different in the $S_2 \rightarrow S_3$ transition where we found the lowest transition efficiency (and highest miss) at all temperatures. Here, EXAFS studies have revealed major structural rearrangements in the CaMn$_4$O$_5$ cluster involving a shift in coordination number for one of the manganese atoms (31, 52–54). In addition, $S_2 \rightarrow S_1$ is pH-dependent and shows the highest deuterium isotope effect on the kinetics for electron transfer from Y$_{Z'}$ (32, 55). Both effects reflect large proton movements both around Y$_Z$ and the CaMn$_4$O$_5$ cluster. Our results clearly suggest that this complicated chemistry is reflected in a lowered transition efficiency.

Very large chemical changes and protein structural rearrangements also take place during the $S_2 \rightarrow S_3$ transition, involving the final step in the water oxidation cycle and the step where O$_2$ is released (31, 34, 52–54). However, as we have mentioned above, this final step in the water oxidation is thermodynamically not very demanding, exothermic, and not limited by the product inhibition of the O$_2$ formation step (48, 49). Probably the structural relaxation reactions take place simultaneously with deuteronation events and O$_2$ release which can explain the higher transition efficiency than in the $S_2 \rightarrow S_3$ transition. The intermediate transition efficiency in the $S_0 \rightarrow S_1$ transition also can be correlated to the chemistry of this transition (35, 50, 51) which is considered to involve deuteronation of a μ-oxo-bridge between two of the manganese atoms (5). This is a smaller structural change than the change in manganese coordination number thought to occur in the $S_2 \rightarrow S_3$ and $S_3 \rightarrow S_0$ transitions.

CONCLUSION

In the present study we are able, for the first time since the discovery of the period four oscillation of the WOC, to measure directly the misses that occur in the WOC in each transition during the catalytic turnover of the WOC. Our results show that the misses are distributed very unevenly in the S cycle. We propose that the fraction of the misses responsible for this difference originates in the reactions at the CaMn$_4$O$_5$ cluster. Experiments to estimate quantitatively this fraction of the miss in each transition under various conditions are underway.

Acknowledgment—We thank Johannes Sjöholm for help in some measurements.

REFERENCES

1. Wydrzynski, T. J., and Satoh, K. (eds) (2005) Photosystem II: The Light-driven Water:Plastoquinone Oxidoreductase. Springer, The Netherlands
2. Renger, G. (2008) in Primary Processes of Photosynthesis (Renger, G., ed) Vol. 2, pp. 237–290, RSC Publishing, Cambridge, UK
3. Rappaport, F., and Diner, B. A. (2008) Primary photochemistry and energetics leading to the oxidation of the Mn$_4$Ca cluster and to the evolution of molecular oxygen in photosystem II. Coord. Chem. Rev. 252, 259–272
4. McEvoy, J. P., and Brudvig, G. W. (2006) Water-splitting chemistry of photosystem II. Chem. Rev. 106, 4455–4483
5. Dau, H., and Haumann, M. (2008) The manganese complex of photosystem II in its reaction cycle: basic framework and possible realization at the atomic level. Coord. Chem. Rev. 252, 273–295
6. Messinger, J., and Renger, G. (2008) in Primary Processes of Photosynthesis (Renger, G., ed) Vol. 2, pp. 291–349. RSC Publishing, Cambridge, UK
7. Kamiya, N., and Shen, J. R. (2003) Crystal structure of oxygen-evolving photosystem II from Thermosynechooccus vulcanus at 3.7Å resolution. Proc. Natl. Acad. Sci. U.S.A. 100, 98–103
8. Ferreira, K. N., Iverson, T. M., Maghlaoui, K., Barber, J., and Iwata, S. (2004) Architecture of the photosynthetic oxygen-evolving center. Science 303, 1831–1838
9. Loll, B., Kern, J., Saenger, W., Zouni, A., and Biesiadka, J. (2005) Towards complete cofactor arrangement in the 3.0Å resolution structure of photosystem II. Nature 438, 1040–1044
10. Umena, Y., Kawakami, K., Shen, J. R., and Kamiya, N. (2011) Crystal structure of oxygen-evolving photosystem II at a resolution of 1.9 Å. Nature 473, 55–60
11. Kok, B., Forbush, B., and McGloin, M. (1970) Cooperation of charges in photosynthetic O$_2$ evolution. I. A linear four step mechanism. Photochem. Photobiol. 11, 457–475
12. Forbush, B., Kok, V., and McGloin, M. (1971) Cooperation of charges in photosynthetic O$_2$ evolution. 2. Damping of flash yield oscillation, deactivation. Photochem. Photobiol. 14, 307–321
13. Styring, S., and Rutherford, A. W. (1988) Deactivation kinetics and temperature dependence of the S state transitions in the oxygen-evolving system of photosystem II measured by electron paramagnetic resonance spectroscopy. Biochim. Biophys. Acta 933, 378–387
14. Chen, G., Han, G., Göransson, E., Mamedov, F., and Styring, S. (2012) Stability of the S$_3$ and S$_2$ state intermediates in photosystem II directly probed by EPR spectroscopy. Biochemistry 51, 138–148
15. Styring, S., and Rutherford, A. W. (1987) In the oxygen-evolving complex of photosystem II the S$_0$ state is oxidized to the S$_1$ state by D$^+$ (signal II$_{ano}$). Biochemistry 26, 2401–2405
16. Joliot, P., Barbieri, G., and Chabaud, R. (1969) Un nouveau modele des centres photochimiques du systeme II. Photochem. Photobiol. 10, 309–329
17. Shinkarev, V. P., and Wraight, C. A. (1993) Oxygen evolution in photosynthesis: from unicyle to bicycle. Proc. Natl. Acad. Sci. U.S.A. 90, 1834–1838
18. Lavergne, J., and Rappaport, F. (1998) Stabilization of charge separation and photochemical misses in photosystem II. Biochemistry 37, 7899–7906
19. Meunier, P. C. (1993) Oxygen evolution by photosystem II: the contribution of backward transitions to the anomalous behaviour of double-hits revealed by a new analysis method. Photosynth. Res. 36, 111–118
20. Shinkarev, V. P. (2005) Flash-induced oxygen evolution in photosynthesis: simple solution for the extended S-state model that includes misses, double-hits, inactivation, and backward-transitions. Biophys. J. 88, 412–421
21. de Wijn, R., and van Gorkom, H. J. (2002) S-state dependence of the miss
probability in photosystem II. *Photosynth. Res.* 72, 217–222

22. Packham, N. K., Hodges, M., Etienne, A. L., and Briantais, J. M. (1988) Changes in the flash-induced oxygen yield pattern by thylakoid membrane phosphorylation. *Photosynth. Res.* 15, 221–232

23. Messinger, J., Schröder, W. P., and Renger, G. (1993) Structure-function relations in photosystem II: effects of temperature and chaotropic agents on the period four oscillation of flash-induced oxygen evolution. *Biochemistry* 32, 7658–7668

24. Messinger, J., and Renger, G. (1994) Analyses of pH-induced modifications of the period four oscillation of flash-induced oxygen evolution reveal distinct structural changes of the photosystem II donor side at characteristic pH values. *Biochemistry* 33, 10896–10905

25. Meunier, P. C., Burnap, R. L., and Sherman, L. A. (1996) Improved 5-step modeling of the photosystem II S-state mechanism in cyanobacteria. *Photosynth. Res.* 51, 67–76

26. Isgandarova, S., Renger, G., and Messinger, J. (2003) Functional differences of photosystem II from *Synechococcus elongatus* and spinach characterized by flash-induced oxygen evolution patterns. *Biochemistry* 42, 8929–8938

27. Lavorel, J. (1976) Matrix analysis of the oxygen evolving system of photosynthesis. *J. Theor. Biol.* 57, 171–185

28. Ahrling, K. A., Peterson, S., and Styring, S. (1997) An oscillating manganese electron paramagnetic resonance signal from the S4 state of the oxygen evolving complex in photosystem II. *Biochemistry* 36, 13148–13152

29. Roelofs, T. A., Liang, W., Latimer, M. J., Cinco, R. M., Rompel, A., Andrews, J. C., Sauer, K., Yachandra, V. K., and Klein, M. P. (1996) Oxidation states of the manganese cluster during the flash-induced S-state cycle of the photosynthetic oxygen-evolving complex. *Proc. Natl. Acad. Sci. U.S.A.* 93, 3335–3340

30. Messinger, J., Robblee, J. H., Bergmann, U., Fernandez, C., Glätzel, P., Visser, H., Cinco, R. M., McFarlane, K. L., Bellacchio, E., Pizarro, S. A., Cramer, S. P., Sauer, K., Klein, M. P., and Yachandra, V. K. (2001) Absence of Mn-centered oxidation in the S4→S3 transition: implications for the mechanism of photosynthetic water oxidation. *J. Am. Chem. Soc.* 123, 7804–7820

31. Haumann, M., Müller, C., Liebisch, P., Iuzzolino, L., Dittmer, J., Grabolle, M., Neisius, T., Meyer-Klaucke, W., and Dau, H. (2005) Structural and oxidation state changes of the photosystem II manganese complex in four transitions of the water oxidation cycle (S4 → S3, S4 → S5, S4 → S6 and S6 → S7) characterized by X-ray absorption spectroscopy at 20 K and room temperature. *Biochemistry* 44, 1894–1908

32. Christen, G., and Renger, G. (1999) The role of hydrogen bonds for the multiphasic P680*+ reduction by YZ in photosystem II with intact oxygen evolution capacity: analysis of kinetic H/D isotope exchange effects. *Biochemistry* 38, 2068–2077

33. Iuzzolino, L., Dittmer, J., Dörner, W., Meyer-Klaucke, W., and Dau, H. (1998) X-ray absorption spectroscopy on layered photosystem II membrane particles suggests manganese-centered oxidation of the oxygen-evolving complex for the S5→S4, S4→S5, and S3→S4 transitions of the water oxidation cycle. *Biochemistry* 37, 17112–17119

34. Pushkar, Y., Yano, J., Sauer, K., Boussac, A., and Yachandra, V. K. (2008) Structural changes in the Mn4Ca cluster and the mechanism of photosynthetic water splitting. *Proc. Natl. Acad. Sci. U.S.A.* 105, 1879–1884

35. Bernát, G., Morvaridi, F., Feyziyev, Y., and Styring, S. (2002) pH dependence of the four individual transitions in the catalytic S-cycle during photosynthetic oxygen evolution. *Biochemistry* 41, 5830–5843

36. Han, G., Ho, F. M., Havelius, K. G. V., Morvaridi, S. F., Mamedov, F., and Styring, S. (2008) Direct quantification of the four individual S states in photosystem II using EPR spectroscopy. *Biochim. Biophys. Acta* 1777, 495–503

37. Havelius, K. G. V., Sjöholm, J., Ho, F. M., Mamedov, F., and Styring, S. (2010) Metalloradical EPR signals from the Y3’S state intermediates in photosystem II. *Appl. Magnetic Resonance* 37, 151–176

38. Petrouleas, V., Koulougliotis, D., and Ioannidis, N. (2005) Trapping of metalloradical intermediates of the S-states at liquid helium temperatures: overview of the phenomenology and mechanistic implications. *Biochemistry* 44, 6723–6728

39. Berthold, D. A., Babcock, G. T., and Yocum, C. F. (1981) A highly resolved, oxygen-evolving photosystem II preparation from spinach thylakoid membranes: EPR and electron-transfer properties. *FEBS Lett.* 134, 231–234

40. Völker, M., Ono, T., Inoue, Y., and Renger, G. (1985) Effect of trypsin on PS-II particles: correlation between Hill activity, Mn abundance, and peptide pattern. *Biochim. Biophys. Acta* 806, 25–34

41. Jeans, C., Schilsitra, M. J., Ray, N., Husain, S., Minagawa, J., Nugent, J. H., and Klug, D. R. (2002) Replacement of tyrosine D with phenylalanine affects the normal proton transfer pathways for the reduction of P680* in oxygen-evolving photosystem II particles from *Chlamydomonas.* *Biochemistry* 41, 15754–15761

42. Saygin, Ö., and Witt, H. T. (1987) Optical characterization of intermediates in the water splitting enzyme system of photosynthesis: possible states and configurations of manganese and water. *Biochim. Biophys. Acta* 893, 452–469

43. Hwang, H. J., Dilbeck, P., Debus, R. J., and Burnap, R. L. (2007) Mutation of arginine 357 of the CP43 protein of photosystem II severely impairs the catalytic S-state cycle of the H2O oxidation complex. *Biochemistry* 46, 11987–11997

44. Joliot, P., and Kok, B. (1975) in *Bioenergetics of Photosynthesis* (Govindjee, ed) pp. 388–413, Academic Press, New York

45. Reimann, S., and Mathis, P. (1981) Influence of temperature on photosystem II electron transfer reactions. *Biochim. Biophys. Acta* 635, 249–258

46. Mamedov, F., Sayre, R. T., and Styring, S. (1998) Involvement of histidine 190 on the D1 protein in electron/proton transfer reactions on the donor side of photosystem II. *Biochemistry* 37, 14245–14256

47. Delrieu, M. J. (1983) Evidence for unequal misses in oxygen flash yield sequence in photosynthesis. *Zeitschrift Naturforschung C* 38, 247–258

48. Haumann, M., Grundmeier, A., Zaharieva, I., and Dau, H. (2008) Photosynthetic water oxidation at elevated dioxygen partial pressure monitored by time-resolved x-ray absorption measurements. *Proc. Natl. Acad. Sci. U.S.A.* 105, 17384–17389

49. Shevela, D., Beckmann, K., Clausen, J., Junge, W., and Messinger, J. (2011) Membrane-inlet mass spectrometry reveals a high driving force for oxygen production by photosystem II. *Proc. Natl. Acad. Sci. U.S.A.* 108, 3602–3607

50. Suzuki, H., Sugita, M., and Noguchi, T. (2005) pH dependence of the flash-induced S-state transitions in the oxygen-evolving center of photosystem II from *Thermosynechococcus elongatus* as revealed by Fourier transform infrared spectroscopy. *Biochemistry* 44, 1708–1718

51. Schlodder, E., and Witt, H. T. (1999) Stoichiometry of proton release from the catalytic center in photosynthetic water oxidation: reexamination by a glass electrode study at pH 5.5–7.2. *J. Biol. Chem.* 274, 30387–30392

52. Robblee, J. H., Cinco, R. M., and Yachandra, V. K. (2001) X-ray spectroscopy-based structure of the Mn cluster and mechanism of photosynthetic oxygen evolution. *Biochim. Biophys. Acta* 1503, 7–23

53. Haumann, M., Grabolle, M., Neisius, T., and Dau, H. (2002) The first room-temperature x-ray absorption spectra of higher oxidation states of the tetramanganese complex of photosystem II. *FEBS Lett.* 512, 116–120

54. Yano, J., Pushkar, Y., Glätzel, P., Lewis, A., Sauer, K., Messinger, J., Bergmann, U., and Yachandra, V. (2005) High-resolution Mn EXAFS of the oxygen-evolving complex in photosystem II: structural implications for the Mn6Ca cluster. *J. Am. Chem. Soc.* 127, 14974–14975

55. Christen, G., Seeliger, A., and Renger, G. (1999) P680*+ reduction kinetics and redox transition probability of the water oxidizing complex as a function of pH and H/D isotope exchange in spinach thylakoids. *Biochemistry* 38, 6082–6092