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THERMOLUMINESCENCE AND OXYGEN EVOLUTION FROM A THERMOPHILIC BLUE-GREEN ALGA OBTAINED AFTER SINGLE-TURNOVER LIGHT FLASHES*

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Abstract—Oxygen evolution and thermoluminescence (TL) studies on a thermophilic blue-green alga *Synechococcus vulcanus* Copeland revealed the following: (a) The deactivations of the S_3 and S_2 states of the Oxygen Evolving Complex, at room temperature, have half-times of ~200 and 75 s, respectively, instead of 30 and 20 s found in mesophilic plants. (b) The TL band(s) "B", due to the recombination of the state S_2 or S_3 and Q_B^-, the reduced secondary quinone acceptor, is(are) at 50–55°C instead of 25–30°C; the intensity of this band oscillates with a period of 4 with maxima on the 2nd and the 6th flashes. (c) The TL band "D" in the presence of diuron, due to the recombination of S_2 and the reduced primary quinone acceptor Q_A^-, occurs at ~35°C instead of 0–10°C. (d) Furthermore, the ratio of Q_B^- to Q_B in dark-adapted *S. vulcanus* cells is close to 1 as in intact spinach leaves, but not 0.43 as in isolated thylakoids from spinach.

INTRODUCTION

Thermophilic algae provide a unique system to study the stability of the various photosynthetic reactions and, one hopes to learn from such studies the means to stabilize such reactions in mesophilic plants. Both photosynthetic carbon fixation rates and electron transport rates in a thermophilic algae *Synechococcus* show maxima at high temperatures (50–60°C) (Yamaoka *et al.*, 1978a; 1978b; Hirano *et al.*, 1981a, 1981b; Aoki and Katoh, 1982). The intrinsic pigment-proteins and water soluble proteins isolated from this alga are resistant to heat treatment; the temperature causing inactivation of these proteins is higher by about 30°C than that for mesophilic plants or algae (Koike *et al.*, 1979, 1981, 1982; Koike and Inoue, 1983). Most plants contain three extrinsic polypeptides of molecular mass 34, 24 and 18 kilodaltons (kDa), associated with the oxygen evolving complex, OEC[1] (see review by Govindjee *et al.*, 1985). However, *Synechococcus vulcanus* Copeland, which grows at 56°C (Koike and Inoue, 1983), appears to be missing both the 18 and 24 kDa polypeptides (Koike and Inoue, 1985). Thus, this system is of unique importance to the study of photosystem II (PSII)[§] reactions especially those dealing with the operation of the OEC, i.e., the "oxygen clock" (Kok *et al.*, 1970), and the recombination reactions involving the S-states of this "oxygen clock" leading to thermoluminescence (see e.g. reviews by Inoue and Shibata, 1982; Inoue, 1983; Sane and Rutherford, 1986).

The OEC operates, according to the S-state hypothesis of Kok *et al.* (1970), as follows (for 75% of OEC):

$$S_1 \xrightarrow{{}^1h\nu} S_2 \xrightarrow{{}^2h\nu} S_3 \xrightarrow{{}^3h\nu} S_4 \xrightarrow{O_2} \qquad S_0 \xrightarrow{{}^4h\nu} S_1 \qquad (1)$$

where S's represent the different redox states of OEC, the subscript representing the oxidizing equivalent, and the superscript before $h\nu$ (photon) represents the number of the flash. A minority (25%) of OEC start out in S_0 in dark-adapted samples. Thus, the O_2/flash shows a maximum in the 3rd and the 7th flashes. The deactivation of the various S-states can be measured by setting the system in specific S-states and by varying the dark-time between flashes. For example, deactivation of S_3 can be measured by varying the dark time after 2 preflashes, and measuring O_2 yield in the following first flash (Y_1); and S_2 deactivation can be measured by varying the dark time after 1 preflash, and measuring O_2 yield in the following second flash (Y_2) with 1 second dark period between the last two flashes.

Thermoluminescence (TL), on the other hand, measures the status of both the S-states of the OEC and the electron acceptor side (see e.g., Rutherford *et al.*, 1982, 1984a; Demeter and Vass, 1984). In mesophilic plants, the TL peak at 25–30°C (also called the "B" band, or peak IV) (Sane and Ruther-

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§*Abbreviations*: Chl, chlorophyll; kDa, kilodalton; OEC, oxygen evolving complex; Diuron (DCMU), 3-(3,4 dichlorophenyl)-1,1'-dimethyl urea; PSII, photosystem II; TL, thermoluminescence.

ford, 1986) originates in $S_2Q_B^-/S_3Q_B^-$ recombination, as described below.

$$S_2 \cdot Z \cdot P680 \cdot Q_A^- \cdot I \cdot Q_B \text{ (species created by the 1st flash)} \xrightarrow{\text{warm}} \quad (2a)$$

$$S_2 \cdot Z \cdot P680 \cdot I \cdot Q_A^- \cdot Q_B \xrightarrow{\text{warm}} \quad (2b)$$

$$S_1 \cdot Z^+ \cdot P680 \cdot I^- \cdot Q_A \cdot Q_B \xrightarrow{\text{warm}} \quad (2c)$$

$$S_1 \cdot Z \cdot P680^+ \cdot I^- \cdot Q_A \cdot Q_B \xrightarrow{\text{warm}} \quad (2d)$$

$$S_1 \cdot Z \cdot P680^* \cdot I \cdot Q_A Q_B \longrightarrow \quad (2e)$$

$$S_1 \cdot Z \cdot P680 \cdot I \cdot Q_A \cdot Q_B + \text{thermoluminescence } (h\nu) \quad (2f)$$

Here, Z refers to the electron donor to the reaction center chlorophyll *a* P680, I to pheophytin, Q_A to the first bound-quinone electron acceptor of PSII, and Q_B to the second bound-quinone electron acceptor of PSII; the latter can accept two electrons whereas Q_A is a one electron acceptor.

When diuron is present, electrons cannot be transferred from Q_A^- to Q_B, and one starts with (2b), and thus one measures the $S_2Q_A^-$ recombination reaction (see e.g., Rutherford *et al.*, 1982). If, however, flash excitation at room temperature is followed by one minute continuous illumination at 77K, then it is possible to change the electron acceptor side without changing the S-state (see e.g., Rutherford *et al.*, 1982) (for simplicity, some of the intermediates, shown in Eq. 2, have been left out):

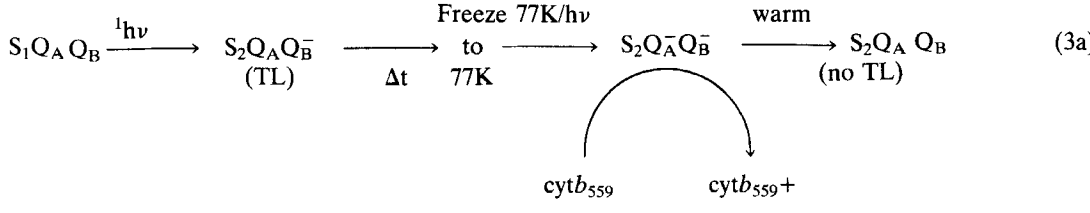
Here, PQ stands for the plastoquinone pool and cyt for cytochrome. If we have an equal ratio of $[Q_B^-]/[Q_B]$ in dark-adapted sample, then the TL intensity with and without illumination at 77K is expected to be the same (see Eqs. 3a and b; Rutherford *et al.*, 1984b). However, if $[Q_B] > [Q_B^-]$, then illumination at 77 K would decrease TL (see Eq. 3a), and if $[Q_B] < [Q_B^-]$, one would observe an increase (see Eq. 3b). By varying the time (Δt in Eq. 3) between flash and cooling to 77 K, the deactivation of S-states can also be measured through the TL method.

In this paper, we present our data on the flash-dependence of TL and O_2 yield as a function of flash number, on deactivation times of S_2 and S_3 states, and on the effects of illumination at 77 K following the flash to monitor the ratio of Q_B/Q_B^- in dark-adapted thermophilic *Synechococcus vulcanus* cells.

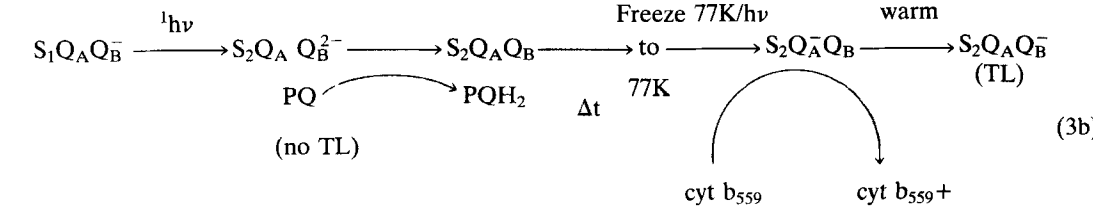
MATERIALS AND METHODS

The thermophilic blue-green alga *Synechococcus vulcanus* Copeland was collected from a hot spring in Wakayama, Japan. The alga was grown at 55°C for 24 h in a medium described by Hirano *et al.* (1981). The culture medium was supplied with CO_2 by bubbling with 3% CO_2-enriched air. The cells were harvested by centrifugation (1500 **g** for 5 min, at 30°C), and suspended in the fresh medium to give a final chlorophyll (Chl) concentration of 1 mg/mℓ, and kept in the dark at 25°C. Chlorophyll concentration was determined by the method of Mackinney (1941).

Thermoluminescence was measured on an instrument of Ichikawa *et al.* (1975) as described by Govindjee *et al.* (1984). The sample, uniformly spread on a 1 × 1 cm filter paper, was placed directly on the heating plate; and the thermocouple was placed on this filter paper. After a long dark-adaptation (20–35 min), the sample was exposed to saturating Xe flashes (5 μs, 4.5 J, white light) at room temperature, cooled to 77 K and then the TL was measured upon warming in the dark as described earlier (Ichikawa *et al.*, 1975; Govindjee *et al.*, 1984). The heating rate was 0.5°C/s. If the deactivation time had to be measured, the sample was kept at fixed times at room temperature before



(3a)



(3b)

being cooled to 77 K. Continuous illumination was provided by tungsten light filtered through a Corning (CS2-58) orange glass filter ($\lambda > 570$ nm).

Oxygen evolution and deactivation of S-states were measured with a Joliot-type electrode as described by Joliot *et al.* (1971). After the cells were dark adapted for 30 min at room temperature, the sample was excited with 5 μs duration Xe flashes, spaced 1 s apart, at room temperature. The sample was changed after each measurement. No electron acceptor was added to the sample.

RESULTS AND DISCUSSION

Figure 1 shows TL of *S. vulcanus* cells measured with or without diuron (DCMU, 10 μ*M*) after illumination with a single flash of light. We ascribe the peak at 55°C to be due to the recombination of $S_2Q_B^-$ produced by the following set of reactions.

$$S_1Q_B \xrightarrow{^1h\nu} S_2Q_B^- \xrightarrow{\text{warm}} S_1Q_B + TL \qquad (4)$$

This peak in mesophilic plants, e.g. spinach, appears at 25–30°C (see Rutherford *et al.*, 1982, 1984a):

When 10 μ*M* diuron (DCMU) is present, the peak is shifted by almost 20°C to the lower temperature because, as noted earlier, we can observe now the $S_2Q_A^-$ recombination. [For theoretical reasons for the shift, see DeVault *et al.* (1983).] When diuron is present, only $S_1Q_A \xrightarrow{h\nu} S_2Q_A^-$ reaction occurs, and produces TL. Thus, the 35°C band with diuron present is not dependent on the flash number. However, the 50–55°C band, due to $S_2Q_B^-$ (after flash 1) as well as to $S_3Q_B^-$ recombination (after other flashes), was highly dependent on the flash number (see below).

Figure 2 shows the 50–55°C band for four flashes (1–4). It is clear that TL intensity after flash 2 is highest, and that the TL peak after flash 1 is at the highest temperature (55°C). After flash 2, the TL peak shifts to a lower temperature (~ 50°C). Both the temperature shift and the change in TL intensity is explained as follows. As discussed later, $[S_0] = 0.20$; $[S_1] = 0.80$; and $[Q_B] = [Q_B^-]$ in dark-adapted *S. vulcanus* cells. Thus, we can write:

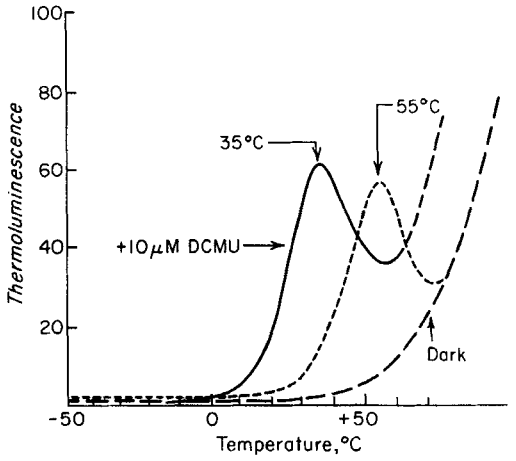


Figure 1. Thermoluminescence (TL; glow) curves of *S. vulcanus* cells. One flash was given to dark adapted cells with (——) and without (---) 10 μ*M* diuron. Dark stands for the TL base line measured without illumination.

In these cells, the number of reaction centers in $S_3Q_B^-$ is 4 times that in $S_2Q_B^-$ after 2 flashes. Since $S_3Q_B^-$ may be more efficient in producing TL (Inoue, 1983; Rutherford *et al.*, 1984c; see later in this paper), its contribution to the observed 50–55°C peak is even higher. Thus, TL after flash 2 is much higher than after flash 1. Furthermore, the peak is at 55°C after flash 1, and at 50°C after flash 2 because the activation energy for $S_2Q_B^-$ recombination is higher than for $S_3Q_B^-$ recombination (see DeVault *et al.*, 1983). To obtain the correct TL intensity at the peak, it is necessary to subtract the "dark" signal (see Fig. 1) which increases with temperature, and can be easily seen beyond 70°C. It is due to non-specific chemiluminescence from the sample (see Sane and Rutherford, 1986). The actual cause of the differences in the "dark" signal is not known, but it includes sample variability. The "light" minus "dark" signal is, however, constant. Thus, these differences in "dark" signals do not affect the picture of the flash pattern.

The insert shows the TL intensities for the 55°C peak as a function of flash number after corrections

$$S_1Q_B \xrightarrow{^1h\nu} S_2Q_B^- \xrightarrow{^2h\nu} S_3Q_B^{2-} \longrightarrow S_3Q_B \quad (40\%) \quad (\text{No TL}) \qquad (5a)$$
$$PQ \quad PQH_2$$

$$S_1Q_B^- \xrightarrow{^1h\nu} S_2Q_B^{2-} \longrightarrow S_2Q_B \xrightarrow{^2h\nu} S_3Q_B^- \text{ (TL) (40\%)} \qquad (5b)$$
$$PQ \quad PQH_2$$

$$S_0Q_B \xrightarrow{^1h\nu} S_1Q_B^- \xrightarrow{^2h\nu} S_2Q_B^{2-} \longrightarrow S_2Q_B \text{ (No TL) (10\%)} \qquad (5c)$$
$$PQ \quad PQH_2$$

$$S_0Q_B^- \xrightarrow{^1h\nu} S_2Q_B^{2-} \longrightarrow S_1Q_B \xrightarrow{^2h\nu} S_2Q_B^- \text{ (TL) (10\%)} \qquad (5d)$$
$$PQ \quad PQH_2$$

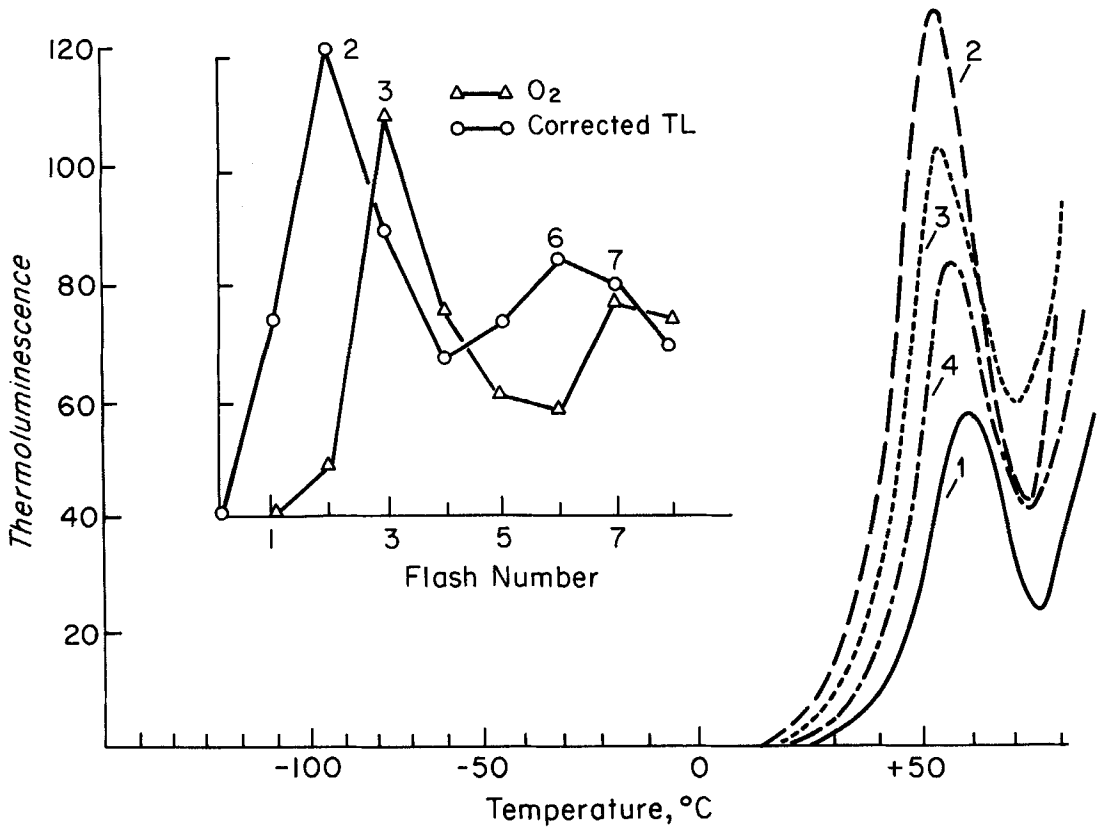


Figure 2. Glow (TL) curves of *S. vulcanus* cells recorded after a series of flashes. Number of flashes given are indicated on each glow curve. Inset: Oscillation of the 53°C band height and O_2 flash yield as a function of flash number.

for TL without any flash (see the curve labeled "Dark" in Fig. 1), and for the general slow increase in TL with time. The plastoquinone (PQ) pool is slowly filled with increasing number of flashes, and this increases the efficiency of the primary back reaction, and, thus a correction is made for increasing TL which is linear with flash number. These corrections, however, had no effect on the positions of the maxima, only on the ratio of the TL at peak 2/TL at peak 6. It is clear that the maxima are on the 2nd and the 6th flashes instead of the 1st and the 5th flashes. The same pattern has been observed in dark-adapted intact leaves (Rutherford *et al.*, 1984a, 1984b), in preilluminated thylakoids (Rutherford *et al.*, 1982), and in dark-adapted intact chloroplasts (Govindjee, T. Ono and Y. Inoue, 1983, unpublished; Demeter and Vass, 1984). For comparison, the insert also shows the O_2 yield/flash as a function of flash number in identical samples; the period 4 oscillations with peaks after the 3rd and the 7th flashes are clearly observed (to be analyzed and discussed below).

The TL peaks after the 2nd and the 6th flashes, instead of the 1st and 5th flashes, in dark adapted (35 min) algal cells are best explained by the assumption that the ratio of Q_B^-/Q_B is higher in these cells than in dark-adapted thylakoids. If this ratio is 1 (see later), then 50% of the centers in S_1 (which itself has a concentration 4 times that of S_0) undergo the reaction:

$$S_1Q_B^- \xrightarrow{^1h\nu} S_2Q_B^{2-} \longrightarrow S_2Q_B \xrightarrow{^2h\nu} S_3Q_B^- \quad \text{(TL)}$$
$$\text{PQ} \quad \text{PQH}_2 \quad \text{(No TL)} \qquad (6a)$$

And, 50% of the centers in S_0 undergo the reaction:

$$S_0Q_B^- \xrightarrow{^1h\nu} S_1Q_B^{2-} \longrightarrow S_1Q_B \xrightarrow{^2h\nu} S_2Q_B^- \quad \text{(TL)}$$
$$\text{PQ} \quad \text{PQH}_2 \quad \text{(No TL)} \qquad (6b)$$

Thus, TL is higher after the second than after the first flash. The above idea that Q_B^-/Q_B ratio is higher in *S. vulcanus* cells, than in isolated thylakoids of higher plants, was confirmed by experiments shown in Fig. 3. As discussed earlier (Eq. 3), continuous light given at 77 K to flash-illuminated samples should change the TL intensity if $[Q_B] \neq [Q_B^-]$. Lack of any significant effect of continuous light illumination at 77 K on the TL intensity after both the 1st and 2nd flashes confirms the idea that $[Q_B] = [Q_B^-]$ in dark-adapted *S. vulcanus* cells. This is consistent with the PQ pool being more reduced in intact cells and responsible for the higher $[Q_B^-]$ (see Rutherford *et al.*

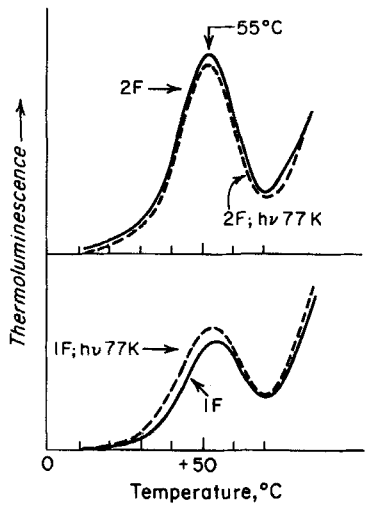


Figure 3. Glow (TL) curves of *S. vulcanus* cells recorded after one (1F) or two flashes (2F) given at room temperature (——) and those followed by 1 min illumination with continuous orange light (λ > 570 nm) at 77 K (---).

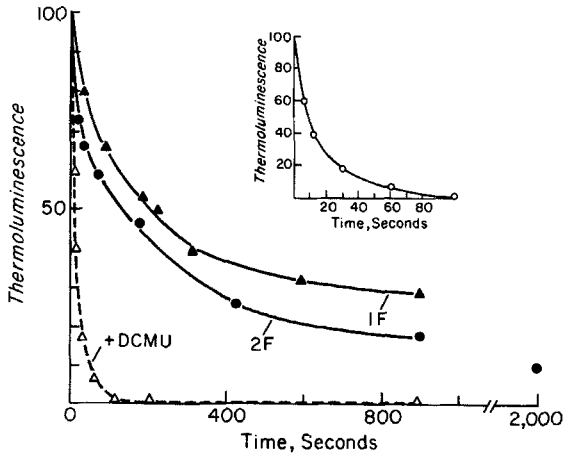


Figure 4. Time-dependent decay curves of TL intensity after flash excitation of *S. vulcanus* cells. Dark-adapted cells were illuminated with one (▲, 1F) or two (●, 2F) flashes and incubated in darkness at room temperature before being frozen. TL intensity was plotted against the time of dark incubation. Inset and (Δ): Decay curve of TL in the presence of 10 μ*M* diuron.

(1984b) for references and further discussion on this topic).

Decay of the S-states, measured by TL after one flash ($S_2Q_B^-$ deactivation) or after two flashes ($S_3Q_B^-$ and $S_2Q_B^-$ deactivation) is shown in Fig. 4. The halftimes are 220 s after flash 1 and 150 s after flash 2. But, $S_2Q_A^-$ deactivation appears to have a halftime of 10 s (see insert in Fig. 4). The $S_2Q_B^-$ and $S_3Q_B^-$ deactivations at room temperature are an order of magnitude slower than in mesophilic plants, where they are 30 and 20 s respectively (Rutherford *et al.*, 1982, 1984).

The time course of the decay of S-states measured by the O_2 flash yield (see e.g. Radmer and Cheniae, 1977) and TL intensities from the same batch of cells are shown in Fig. 5. A semilogarithmic plot of S-state deactivation gives straight lines for both S_3 and S_2 in the first 6 min which give half times of 75 and 204 s, respectively. Although the S_2 deactivation times from both TL (207 s) and O_2 measurements (204 s) are in agreement, the deactivation for S_3 by the TL decay (105 s) appears longer than O_2 method (75 s) (see also Table 1). Since the TL intensity depends upon the concentration of both $[S_2Q_B^-]$ and $[S_3Q_B^-]$, the population of $[S_2]$ cannot be neglected after 2 flashes and thus the decay of TL after two flashes is affected by the concentration of both S_2 and S_3. We

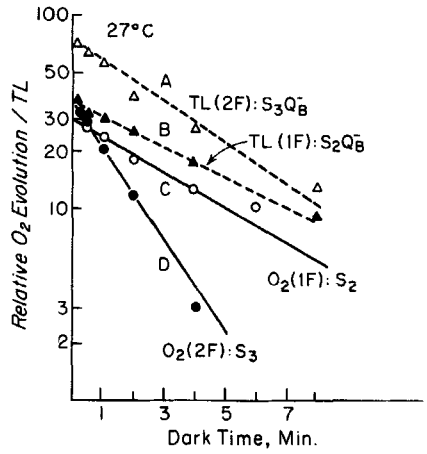


Figure 5. Semilogarithmic plot of the time-dependent decay curves of TL intensity (---) and those of S_2 and S_3 states measured by Joliot-type elctrode (——). (A) TL intensity after two flashes; (B) TL intensity after one flash; (C) O_2 evolution by the second detecting flash after preillumination with one flash; (D) O_2 evolution by the first detecting flash after preillumination with two flashes.

have calculated the TL intensity as a function of time after preillumination with two flashes from the relative population of S_3 and S_2, and from the decay times of S_3 and S_2 (obtained from O_2 data) assuming

Table 1. Comparison of half times of TL intensity and flash O_2 yield in *S. vulcanus* cells

	Half decay time (s)	
Measurement	1 preflash	2 preflashes
O_2 Flash yield	204 (S_2 decay)	75 (S_3 decay)
TL intensity	207 ($S_2Q_B^-$ decay)	150 ($S_3Q_B^-$+$S_2Q_B^-$ decays)

that their decays are first order (see Fig. 5C and D). In these calculations, we assumed two alternate pathways for deactivation: (**I**) S_3 deactivates via S_2 to S_1 where $[Q_B^-]$ does not significantly change during deactivation, and (**2**) both $S_3Q_B^-$ and S_2Q_B deactivate by the recombination process assuming that decay rates of S_3 and S_2, measured by the O_2 method, are determined by the recombination rate. (Using $[S_0]$ = 0.20, $[S_1]$ = 0.80, misses (α) = 0.12 and double hits (β) = 0.07 for dark-adapted cells, we can obtain the following values for S states after 2 flashes: $[S_0]$ = 9.46%, $[S_1]$ = 5.43%, $[S_2]$ = 29% and $[S_3]$ = 56.1%.

In the case of process (**I**), TL intensity (calculated results are shown in Fig. 6(a), curves a, b and c) depends mainly on the relative concentration of S_3 and/or S_2, since the concentration of Q_B^- is almost 50%, and stays almost constant during deactivation. Then, we can calculate the decay of S_2 and S_3 concentrations as follows.

$$\text{If } S_3 \xrightarrow[k_3]{} S_2 \xrightarrow[k_2]{} S_1, \quad (7a)$$

then, $k_3=(\ln 2)/75\ s^{-1}$; and $k_2=(\ln 2)/204\ s^{-1}$ (7b)

from the O_2 experiments (Fig. 5);

$$\frac{d[S_3]}{dt} = -k_3\,[S_3];\ [S_3] = [S_3]_0\, e^{-k_3 t} \quad (7c)$$

where $[S_3]_0$ = concentration of S_3 at time 0; and,

$$\frac{d[S_2]}{dt} = k_3[S_3]-k_2[S_2] = k_3[S_3]_0 e^{-k_3 t}-k_2[S_2]. \quad (7d)$$

Here, the concentration of S_2 was calculated point by point.

In the case of process (**2**), $S_2Q_B^-$ recombines to give S_1Q_B and $S_3Q_B^-$ recombines to give S_2Q_B which is TL silent; then, if S_2Q_B deactivates to S_1Q_B, it does not participate in TL. Thus, the decay of TL intensity can be calculated (see curves a', b' and c' in Fig. 6(a)) by the linear combination of the decay of $[S_3Q_B^-]$ and $[S_2Q_B^-]$. Again, the concentration of Q_B^- is almost 50% of total Q_B after 2 flashes (see Fig. 3), and TL intensity depends mainly on the linear combination of $[S_3]$ and $[S_2]$. Thus, we can calculate the decay of S_3 and S_2 concentration according to Eqs. 7c and 7e, respectively.

$$d[S_2]/dt = -k_2[S_2];\ [S_2] = [S_2]_0\, e^{-k_2 t} \quad (7e)$$

Finally, the relative intensity of TL as a function of time was calculated with the assumption that [TL] = $n[S_3]$ + $[S_2]$, with (a) n = 2; (b) n = 1; or (c) n = 0.5. Figure 6(a) shows the time courses of the calculated decays of TL, with the experimental data normalized to the value at t = 0. The experimental data fit well only with the decay course of TL calculated by assuming, as was done by Rutherford *et al.* (1984c) for chloroplasts, that TL = $2[S_3]$ + $[S_2]$, and that $S_3Q_B^-$ deactivates to $S_2Q_B^-$ without any significant loss of Q_B^- during the first 6 min decay. However, we do not yet know whether $S_2Q_B^-$ deactivates to $S_1Q_B^-$ or recombines to give S_1Q_B.

Figure 6(b) shows the same calculated decay curves of TL and the experimental data after preillumination by one flash. These calculated intensities of TL were almost identical, because the concentration of S_3 is so low that its contribution to TL is negligible. For this reason, the decay curve of TL fits that of S_2 (calculated from O_2 data) without complicated calculations.

CONCLUDING REMARKS

The present work has demonstrated that both $S_2Q_B^-$ and $S_3Q_B^-$ recombination/deactivation reactions having halftimes of 204 and 75 s respectively, as obtained from both TL and O_2 data, are almost ten times slower in thermophilic than in mesophilic

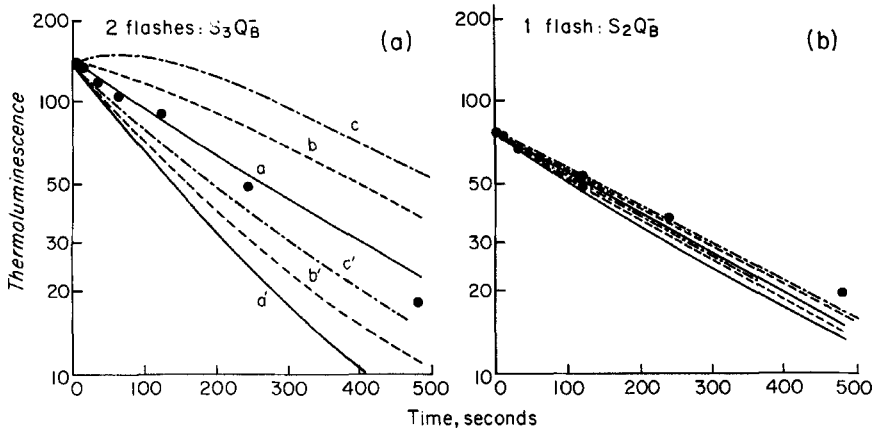


Figure 6. Calculated time-dependent decay curves of TL intensity after two (a) or one (b) preflash(es). TL intensities were calculated by the following assumptions: a,a': TL = $2[S_3]+[S_2]$; b,b': TL = $[S_3]+[S_2]$; c,c': TL = $[S_3]+2[S_2]$. a,b,c: calculated by the process (**I**) (see text); a', b', c': calculated by the process (**2**) (see text) in two preflash decay curves (left Fig. a). One preflash decay curves are shown on the right (Fig. b). Solid circles are the observed decay of TL (*cf.* A and B in Fig. 5). For details see text.

plants at room temperature. On the other hand $S_2Q_A^-$ recombination/deactivation reaction, although slower than in chloroplasts of higher plants, has still a $t_{\frac{1}{2}}$ of ~ 10 s. Thus, the stability of PSII at room temperature in the thermophilic blue-green algae may be due mostly to the stability of $S_2Q_B^-$ and $S_3Q_B^-$ reactions. The relatively high ratio of $[Q_B^-]$:$[Q_B]$, about 1, in the intact cells of *S. vulcanus* is consistent with such ratios in other intact systems (Rutherford *et al.*, 1984b).

An important point to make is that the deactivation experiments performed here have been made 30°C below the temperature at which the recombination of $S_3Q_B^-$ or $S_2Q_B^-$ occurs. Here deactivation, at least of S_3 to S_2, seems to occur also without the loss of Q_B^- (i.e. recombination). We speculate that similar results may be obtained in mesophilic plants if deactivation of S-states was measured at 0–5°C, instead of at room temperature.

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