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Short communication

Flash reactivation of Tris-inactivated chloroplasts

Takashi Yamashita[1], Yorinao Inoue[2], Yoshichika Kobayashi[2] and Kazuo Shibata[2]

[1] Institute of Biological Sciences, University of Tsukuba, Ibaraki 300-31, Japan and
[2] Laboratory of Plant Physiology, The Institute of Physical and Chemical Research, Wako-shi, Saitama 351, Japan

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The light conditions required for reactivation of the oxygen-evolving system in Tris-treated chloroplasts were studied by means of repetitive flashes. Inactive Tris-treated chloroplasts were washed with reduced 2,6-dichlorophenolindophenol, suspended in a reactivation medium containing Mn^{2+} and Ca^{2+} ions, then illuminated with flashes. Flashes at dark intervals of 2 sec were most effective for reactivation, while those at shorter or longer intervals were less effective. It was deduced that more than two sequential light reactions with dark reactions in between were involved in the reactivation.
Key words: Flash photoactivation — Oxygen evolution — Tris-treated chloroplasts.

Light is essential for photosynthetic oxygen evolution not only as a source of energy to oxidize water but also as a stimulus to activate the system for oxygen evolution. The latent inactive system in dark-grown gymnosperm leaves or algal cells or in angiosperm leaves greened with flashes at dark intervals longer than 30 sec requires continuous light or short interval flashes of the order of a few seconds for activation (*1, 4, 6, 8, 9*). The light activation has been shown to involve more than two photoreactions (*5, 10*), but the biochemical reactions involved in the process has remained unknown.

A procedure was recently established to observe the light activation for isolated chloroplasts. Yamashita et al. (*14*) succeeded in reactivating the system in isolated chloroplasts pre-treated with Tris for inactivation. Using this procedure, we can study the light-requiring process involved in the reactivation. The present study deals with the reactivation of Tris-treated chloroplasts by flashes.

The experimental conditions were essentially the same as described previously (*12–14*). Isolated chloroplasts were treated with Tris (0.8 M) at pH 8.8 for 60 min, washed with reduced DCIP (2,6-dichlorophenolindophenol, 0.3 mM) for 20 min, then suspended in a reactivation medium containing 15 mM Tris (pH 7.8), 4 mM $MgCl_2$, 20 mM NaCl, 0.05 mM Mn^{2+}, 2 mM Ca^{2+} and 0.5 mM dithiothreitol at a chlorophyll concentration of 100 μg/ml. Two ml of the chloroplast suspension spread 1.6 mm thick in a Petri dish (4 cm in diameter) was illuminated repeatedly with Xe flashes at a saturating intensity of 2×10^2 ergs/cm^2/flash. Oxygen-evolving activity was measured with a Clark-type oxygen electrode in 1 ml of reaction mixture con-

taining chloroplasts (100 μg chlorophyll), 15 mM Tris (pH 7.8), 4 mM $MgCl_2$, 20 mM NaCl, 2 mM NH_4Cl, 0.05% bovin serum albumin and 2 mM ferricyanide as an electron acceptor, and the activities before and after flash illumination were compared.

Fig. 1 shows the courses of oxygen evolution. The activity of normal chloroplasts was about 100 μmoles O_2/mg Chl·hr, while that of the Tris-treated chloroplasts washed with reduced DCIP but not illuminated was as low as 8.5 μmoles O_2/mg Chl·hr (curve A). When these treated chloroplasts were exposed to one or two hundred flashes repeated at intervals (Td) of 0.5 sec, the activity was recovered slightly but distinctly as shown by curves B and C. On further illumination, the activity increased progressively (curve D to F) the maximum rate shown by curve G, which was obtained after 20-min illumination with continuous light. The yield of such light reactivation varied depending on the preparation of the Tris-treated chloroplasts, but the relative dependence of the reactivation yield on the number of flashes given was reproducible for different preparations. The rate of oxygen evolution was determined after correction by drawing on the traces, as indicated in Fig. 1, for the drift of the base lines (broken lines).

Flash-reactivation experiments similar to the Fig. 1 experiment were carried out under flashes repeated at various intervals to see the effect of the dark interval on the reactivation yield. The four curves in Fig. 2 show the courses of reactivation at the

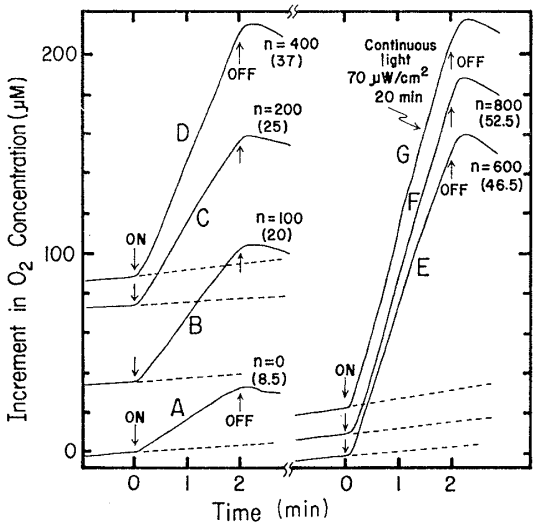

Fig. 1.

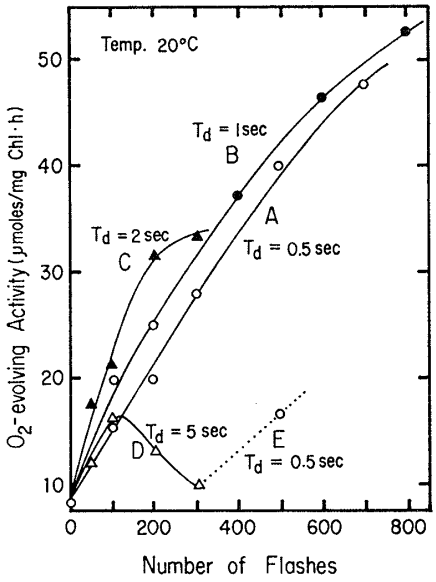

Fig. 2.

Fig. 1. *Courses of oxygen evolution by flash-reactivated chloroplasts.* Curve A shows the course for Tris-treated then DCIP-washed chloroplasts, and curves B to G are the courses for Tris-treated chloroplasts after exposure to different numbers of flashes (n) repeated 0.5 sec apart. Curve G shows the course of the chloroplasts maximally reactivated by continuous illumination with dim room light for 20 min. The rate of oxygen evolution was estimated after correction for the drift of the base line (broken lines), and indicated on each trace in parentheses in μmoles O_2/mg Chl·hr.

Fig. 2. *Light reactivation of the oxygen-evolving activity in Tris-inactivated chloroplasts.* Tris-treated chloroplasts were washed with reduced DCIP then illuminated with flashes repeated at uniform intervals (Td). The activities recovered were plotted against the number of flashes given. The dotted line indicates the activity generation when the flash interval, Td, was changed from 5 to 0.5 sec.

intervals (Td) of 0.5, 1, 2 and 5 sec; the rates of oxygen evolution measured after flash illumination were plotted as a function of the number of flashes given. When the interval was 1 sec or shorter, the activity increased to 50 μmoles O_2/mg Chl·hr as the flash number increased to 700 or 800 (curves A and B). This recovered activity was about 60% of the original activity of the non-treated normal chloroplasts and close to the 56 μmoles O_2/mg Chl·hr obtained by 20-min illumination with continuous dim room light (70 μw/cm^2) (ref. *14*). At longer intervals of Td=2 or 5 sec, however, the activity first increased with increasing flash number then decreased, showing a maximum at what we called the critical flash number (curves C and D).

Fig. 3 shows the effects of the dark interval on the efficiency of reactivation. In the experiment, chloroplasts were illuminated with 100 flashes at uniform intervals, and the interval was varied between 0.1 and 20 sec to observe its effect on the extent of reactivation. In order to avoid disturbance by thermal denaturation of the chloroplasts during prolonged flash illumination, the number of flashes was limited to less than 100, although the activity increment brought about by 100 flashes was as low as 10 μmoles O_2/mg Chl·hr. In spite of the low activities measured, the results reproducibly showed a bell-shaped curve with a maximum at 2 sec. The flashes with longer or shorter intervals were less effective. This is consistent with the previous reactivation mechanism deduced from observation of intact cells and leaves (*1*, *5*, *7*, *10*). The mechanism involves more than two light reactions with a slow dark reaction in between:

$$A \xrightarrow{\text{light}} B \xrightarrow{\text{dark } (\tau_a)} C \xrightarrow{\text{light}} D \qquad \tau_a = 0.3 \text{ sec}$$
$$\xleftarrow[\text{dark } (\tau_b)]{} \qquad \tau_b = 6 \text{ sec}$$

The steep rise of reactivation efficiency at intervals shorter than Td=2 sec indicates that the intermediate (B) formed by the first flash must be converted in the follow-up dark reaction with a rate constant of about 0.3 sec (τ_a) to a light-sensitive state or substance (C), which is to be further converted by the second flash into the final active state (D). The gradual decrease at longer intervals, on the other hand, indicates that the light-sensitive state (C) decays in darkness back to the initial state (A) with a rate constant of about 6 sec (τ_b). The above rate constants, τ_a and τ_b, were roughly estimated from the upward and downward slopes of the curve shown in Fig. 3.

The effect of temperature on the flash reactivation was investigated by changing both temperature and flash interval. The results in Table 1 show the activity increments with the critiacl flash numbers for maximal reactivation in parentheses. At 20°C, maximal reactivation was obtained at Td=2 sec as shown in Fig. 3. At a higher temperature of 30°C, the increment was highest at Td=0.5 sec and decreased steeply at longer intervals whereas, at a lower temperature of 15°C, the increment was slightly higher at Td=2 sec than at Td=0.5 sec. Both dark reactions in the above scheme from B to C and from C back to A are expected to be accelerated at the higher temperature of 30°C. The fact that the efficiency at 30°C was higher at the shorter interval of Td=0.5 sec implies that the acceleration of the former reaction was stronger to overcome the acceleration of the latter back reaction, leading to a higher yield of the final product D. From the rates at the three temperatures at Td=0.5 sec, the Q_{10} value was calculated to be about 2.

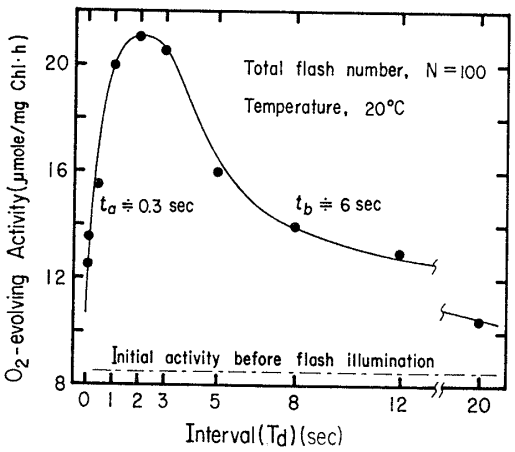

Fig. 3.

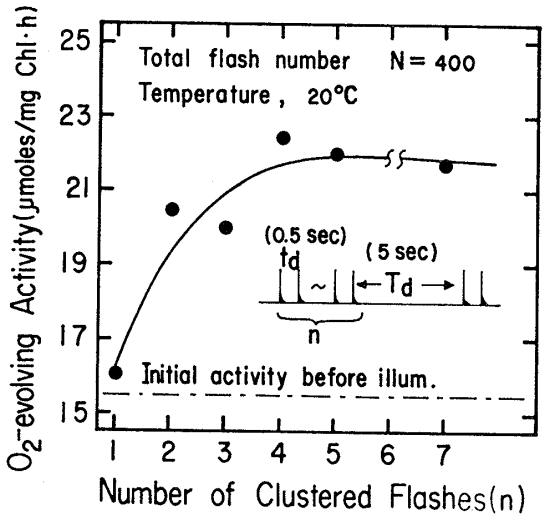

Fig. 4.

Fig. 3. *Effect of the dark interval on light reactivation.* Inactivated chloroplasts were illuminated with 100 flashes repeated at uniform dark intervals, and the activities generated were plotted against the dark interval which was changed between 0.1 and 20 sec.

Fig. 4. *Light reactivation by flash clusters.* Inactivated chloroplasts were illuminated with a total of 400 flashes grouped in flash clusters. The interval between flashes in a cluster was Td=0.5 sec and the interval between clusters was Td=5 sec. The activities generated were plotted against the number (n) of flashes in a cluster.

As seen from Table 1, the critical flash number became smaller as the temperature rose or the interval (Td) increased. The activity first increased then decreased the chloroplasts were illuminated over the critical flash number with the long interval of Td=5 sec (open triangles in Fig. 2). When such chloroplasts were further exposed to the flashes at short optimum intervals of Td=0.5 sec, the activity was enhanced again appreciably (dotted line E in Fig. 2). This suggests that the activity drop above the critical flash number was not simply due to denaturation of the chloroplast structures. We may have to assume a light-dependent deactivation process to account for this phenomenon. Interestingly, this phenomenon has not been reported for the reactivation of the oxygen-evolving system in whole cells (*1, 5, 7*).

The above view with two sequential light reactions was confirmed by the ex-

Table 1 *Dependence of the activity (μmoles O_2/mg Chl·hr) regenerated by 100 flashes on temperature and flash interval (Td)*

Temperature	Activity increment by 100 flashes (μmoles O_2/mg Chl·hr)		
	Td=0.5 sec	Td=2 sec	Td=5 sec
15°C	5 (>600)	8 (>600)	—
20°C	7 (>700)	12 (300)	8 (100)
30°C	11 (400)	7 (200)	2 (—)

The critical flash number is given in parentheses.

periment of Fig. 4, in which chloroplasts were illuminated with flash clusters at saturating intensity. Each cluster was made up of a certain number (n) of flashes at short uniform intervals of 0.5 sec (Td), and the cluster was repeatedly given to the chloroplasts at intervals of 5 sec (Td). The total flash number (N) was fixed at 400 and the flash number (n) for a cluster was varied, so that the cluster was repeated $400/n$ times. The activity generated by these flash clusters was plotted against n in Fig. 4. The reactivation efficiency was nearly zero when $n=1$, appreciably increased between n=2 and 3, and reached a plateau above $n=4$. The fact that the cluster of $n=4$ or more was more efficient than one of $n=2$ or 3 seems to indicate that at least two or more light reactions are involved. The low yield of reactivation and the critical flash number for maximal activation at longer intervals of Td made it difficult to obtain more precise, conclusive data.

The present study confirmed that the light reactivation of the oxygen-evolving system in isolated chloroplasts requires a multi-quantum process similar to that observed for photoactivation of the latent oxygen-evolving system in algal cells and intact leaves (*1*, *5*, *7*). The procedure used here with isolated chloroplasts can offer further insight into the biochemical background of the multi-quantum process, which has been suggested by Cheniae and Martin (*2*, *3*) to be related to the incorporation of Mn^{2+} into thylakoid membranes.

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