ESTIMATION OF THE ACTIVATION ENERGY FOR MILLISECOND DELAYED FLUORESCENCE FROM UNCOUPLED CHLOROPLASTS

M. F. HIPKINS and J. BARBER
Botany Department, Imperial College, London, SW7, England

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1. Introduction

The mechanism that leads to delayed fluorescence from oxygen-evolving photosynthetic systems is thought to involve a back reaction between the reduced primary electron acceptor Q^- and primary electron donor Z^+ of photosystem two [1]. It has become clear from the work of Mayne [2,3] and subsequent workers [4,5] that the high-energy state of phosphorylation acts on this back reaction. This effect is most readily observed in the 1 msec region of the decay. Evidence has been presented that both the electrical ($\triangle\psi$) and chemical ($\triangle$ pH) parts of Mitchell's high-energy state [6] are able to reduce the activation barrier of the back reaction. Direct evidence for the involvement of $\triangle\psi$ in chloroplasts has been shown by Barber [7,8] and Arnold and Azzi [9], and in bacteria by Fleischman [10] and Evans and Crofts [11]. The effect of $\triangle$pH on msec delayed fluorescence from chloroplasts was suggested by Wraight and Crofts [4] and now has experimental support [12] while such an effect has not been found for bacterial delayed fluorescence [11].

For chloroplasts the observations can be summarised in the following expression [13]:

$$J = (Z^+)\,(Q^-)\,\nu\,k'\exp(-(E_{ac} - \Delta p)/kT) \qquad (1)$$

where J is the rate of chlorophyll singlet formation via the back reaction, (Z^+) and (Q^-) are the concentrations of the primary photoproducts, ν is a frequency factor, k' is a constant containing entropy terms, E_{ac} is the activation energy of the back reaction and Δp is the high-energy state or proton motive force (pmf) given by

$$\Delta p = \Delta\psi + 2.303\,\frac{RT}{F}\,\Delta\,\mathrm{pH} \qquad (2)$$

where $\triangle\psi$ is the electrical gradient and $\triangle$ pH is the pH gradient across the thylakoid membrane. J is derived from the eq.

$$L = \phi J \qquad (3)$$

where L is the intensity of delayed fluorescence and ϕ is the prompt fluorescence yield [1,14].

The terms of eq. 1 are not all independent, but it does nevertheless show the involvement of the high-energy state in reducing the activation energy E_{ac}, a complication which clearly perturbs the measurement of E_{ac} and which has been neglected in earlier temperature studies of msec delayed fluorescence [15,16]. The object of the work presented in this paper was to measure E_{ac} in the absence of the high-energy state by the T-jump method [17,18] and discuss the results in terms of the concepts presented above.

2. Materials and methods

Isolation of osmotically broken spinach chloroplasts was essentially the same as described previously [8] except that the chloroplasts were washed and suspended in a medium which contained 0.33 M sucrose, 50 mM KCl and 20 mM N–Tris (hydroxy-methyl)-methyl-2-aminoethane-sulphonic acid (TES) brought to pH 7.0 with KOH. Chlorophyll concentrations were determined by the method of Arnon [19]. Prior to experimenting an appropriate quantity of stock chloroplasts was diluted with the above suspending medium to give a final chlorophyll concentration of 8 μg ml^{-1}. The intensity of 1 msec delayed fluorescence and prompt fluorescence were measured as described earlier [8].

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Temperature changes in the cuvette were monitored with a calibrated copper-constantan thermocouple connected to a Honeywell chart recorder.

Addition of gramicidin was made by injecting 100 μl of the appropriate stock through a light-tight diaphragm. Rapid addition of isothermal or hot suspending medium was accomplished with a syringe inserted through the same diaphragm. The addition of 1 ml of suspending medium to 3 ml of chloroplast suspension was found to give reproducible artifact-free mixing.

3. Results and discussion

It can be seen from eq. 1 that if a suspension of preilluminated chloroplasts is subjected to a sudden increase of temperature then the intensity of delayed fluorescence should increase. Such a stimulation by a T-jump was first observed by Mar and Govindjee [20] and has since been studied in some depth for 10 sec [17] and 0.5 to 1 sec [18] delayed fluorescence. As shown in fig. 1 the emission from isolated chloroplasts in the 1 msec region of the decay is also stimulated by a T-jump. For the reasons given above we have concerned ourselves with the uncoupled state.

Fig. 1A shows the effect of an isothermal addition of suspending medium on prompt and msec delayed fluorescence. The chloroplast suspension contained no electron acceptor and was preilluminated for 1 min such that the maximum level of prompt fluorescence had been reached (that corresponding to the level in the presence of 3-(3,4-dichlorophenyl)-1, 1-dimethylurea (DCMU) where all the photosystem two traps are closed). The shutter across the photomultiplier recording the msec delayed fluorescence was opened as indicated by O and gramicidin was injected into the cuvette. On addition of the uncoupler the intensity of the delayed fluorescence dropped to a low value. The isothermal addition of suspending medium lowered both prompt and delayed fluorescence and from a number of experiments of this type it was established that the relative changes in both the recordings were the same. This was taken to indicate that the stimulation of delayed fluorescence by a T-jump should be corrected for this decrease and that in the case of the isothermal changes the correction was a purely geometric one due to dilution. Fig. 1B shows a typical T-jump experiment carried out in an identical

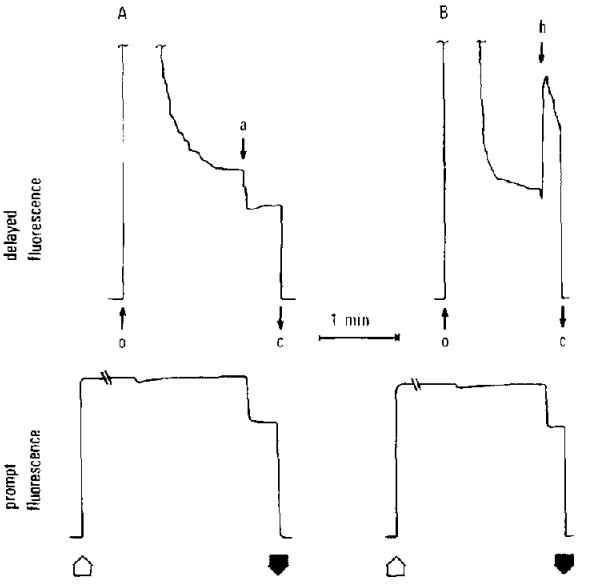

Fig. 1. The effect of an isothermal addition (A) and a temperature jump (B) on the prompt and msec delayed fluorescence from uncoupled broken spinach chloroplasts. The suspension was illuminated for 1 min before the shutter across the delayed fluorescence photomultiplier was opened. The chloroplasts were then uncoupled with 10^{-7} M gramicidin (for the convenience of scale the initial delayed fluorescence level and injection of gramicidin are not shown) and subjected to a rapid addition of 1 ml of isothermal (a) or hot (b) suspending medium. The temperature change due to the hot addition was from 20°C to 34.5°C, while the isothermal experiment was carried out at 20°C. Chlorophyll concentration 8 μg ml^{-1}. Open arrows: light on, closed arrows: light off. The opening and closing of the photomultiplier shutter are indicated by o and c respectively.

manner to the isothermal experiment except there was a change from 20° to 34.5°C at point b. Table 1 gives the results of a series of experiments of this type. The temperature ranges were kept fairly restricted to avoid problems of thermal denaturation and damage of the photosystem two reaction centres.

Bearing in mind that $\Delta p = 0$ and ignoring for the moment any temperature sensitivity of the (Z^+), (Q^-), ν and k' terms of eq. 1, it is possible to calculate E_{ac}

$$E_{ac} = 2.303\, k \frac{T_1 T_2}{T_2 - T_1} \log \frac{J_2}{J_1} \qquad (4)$$

Table 1
Activation energies from T-jumps

T_1 (°C)	T_2 (°C)	$T_2 - T_1$ (°C)	J_2/J_1	E_{ac}(eV)
	13.6	9.6 ± 0.4	2.18 ± 0.09	0.56 ± 0.04
	15.5	11.5 ± 0.5	2.77 ± 0.06	0.61 ± 0.03
4	18.3	14.3 ± 0.2	3.41 ± 0.21	0.60 ± 0.03
	25.1	21.1 ± 1.0	4.79 ± 0.20	0.53 ± 0.03
	21.1	6.1 ± 0.3	1.56 ± 0.05	0.53 ± 0.03
	23.9	8.9 ± 0.4	2.46 ± 0.14	0.75 ± 0.03
15	28.9	13.9 ± 0.6	3.47 ± 0.13	0.69 ± 0.04
	29.2	14.2 ± 0.5	4.47 ± 0.09	0.79 ± 0.03
	27.8	6.3 ± 0.6	1.77 ± 0.04	0.69 ± 0.07
	31.7	10.2 ± 0.3	2.43 ± 0.05	0.68 ± 0.03
21.5	33.1	11.6 ± 0.7	2.63 ± 0.06	0.65 ± 0.04
	34.6	13.1 ± 1.0	3.55 ± 0.02	0.76 ± 0.07

Each J_2 J_1 ratio is the mean of six separate experiments, and the standard errors have been computed from the standard errors of the temperature increase (monitored with a thermocouple) and the standard errors of the delayed fluorescence ratios.

where J_1 and J_2 are the observed intensities of msec delayed fluorescence corrected for the prompt fluorescence yield changes before and after a temperature change from T_1 to T_2.

By obtaining J_2 from the peak of the T-jump signal and T_2 from the thermocouple signal, values of E_{ac} have been calculated using eq. 4 and are presented in table 1. The weighted mean for these values of E_{ac} over the whole temperature range studied is 0.64 eV. This value agrees well with the value of 0.68 ± 0.08 eV recently published for 0.5 to 1 sec delayed fluorescence [18] but does not agree with previous estimates of the activation energy barrier for one msec delayed fluorescence [15,16]. From the earlier work it was reported that the barrier for the msec emission process was much smaller, a conclusion which can probably be partly accounted for both by the presence of Δp and its temperature sensitivity.

The limitation of the above approach is that it has assumed that all other processes are temperature independent. Our calculations have to some extent allowed for temperature induced changes in fluorescence yield and, since there was no net electron flow occurring with our preparations, variations in fluorescence yield due to changes in the redox state of the system two traps were avoided. The analysis may be made more rigorous by using transition state theory to show that the frequency factor ν of eq. 1 is a linear function of temperature and as a consequence introduces a factor $\log (J_2 T_1 / J_1 T_2)$ instead of $\log (J_2/J_1)$ into eq.4. Such a correction decreases the above value by about 4% to 0.61 eV. It has been assumed also that the formation and non-radiative degradation of the precursors Z^+ and Q^- are temperature insensitive and that their concentrations do not change during the T-jump. There is no way to check or correct for these effects because of the nature of the experiments and the methods used, but Malkin and Hardt [18] have emphasised the necessity for such corrections which they were able to make for T-jump measurements at longer dark times.

Bearing in mind the above objections and errors which are inevitable for this type of measurement our results do clearly show, in contradistinction to earlier work [15,16] that msec delayed fluorescence does result from a back reaction involving a significant energy barrier. The corrected value of E_{ac} compares reasonably well with that reported by Malkin and Hardt [18] but unfortunately their elegant analysis was not applied to studies with uncoupled systems and failed to make allowances for time dependent changes in prompt fluorescence yield, factors which are probably signifi-

cant for the part of the decay that they investigated (0.5–1 sec). Accepting that msec delayed fluorescence results from a back reaction in the photosystem two reaction centre and realising that more than one oxidation state of Z is probably involved [21,22] then a value for E_{ac} of 0.61 eV indicates that about 1.19 eV can be extracted from the 1.8 eV available from the singlet excited chlorophyll for driving primary reactions in photosynthesis, a value which is consistant with theoretical arguments [13].

Finally it is worth noting that eq. 1 can be used to estimate the significance of Δp on the emission process. For a coupled system eq. 1 simplifies to

$$J \text{ coupled} \propto \exp(-E_{ac} - \Delta p / kT) \quad (5a)$$

while for the uncoupled state

$$J_{\text{uncoupled}} \propto \exp(-E_{ac}/kT) \quad (5b)$$

Taking the ratio gives

$$\frac{J_{\text{coupled}}}{J_{\text{uncoupled}}} = \exp(\triangle p/kT) \quad (6)$$

If Δp is of the order of 200 mV or more as expected (6) then the ratio J coupled /J uncoupled would be in excess of 2000. In fact such a large ratio is not observed. Usually we find that in the absence of electron acceptors the ratio is at most 15, corresponding to about 70 mV while in the presence of electron acceptors the ratio never exceeds 100, a value which gives a maximum Δp of 120 mV. These simple considerations suggest either that only one component of Δp acts on the back reaction ($\triangle\psi$ or $\triangle$ pH), or that the influence of the total pmf is reduced because of the positioning or orientation of the precursor complex in the membrane.

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References

[1] Lavorel, J. (1968) Biochim. Biophys. Acta 153, 727–730.
[2] Mayne, B. C. (1967) Photochem. Photobiol. 6, 189–197.
[3] Mayne, B. C. (1968) Photochem. Photobiol. 8, 107–113.
[4] Wraight, C. A. and Crofts, A. R. (1971) European J. Biochem. 19, 386–397.
[5] Neumann, J., Barber, J. and Gregory, P. (1973) Plant Physiol. 51, 1069–1073.
[6] Mitchell, P. (1966) Biol. Rev. 41, 445–502.
[7] Barber, J. (1972) FEBS Letters 20, 251–254.
[8] Barber, J. (1972) Biochim. Biophys. Acta 275, 105–116.
[9] Arnold, W. A. and Azzi, J. (1971) in Biomembranes (Manson, L. A., ed), vol. 2, pp. 189–191, Plenum, New York.
[10] Fleischman, D. E. (1971) Photochem. Photobiol. 14, 277–286.
[11] Evans, E. H. and Crofts, A. R. (1974) Biochim. Biophys. Acta 333, 44–51.
[12] Evans, E. H. and Crofts, A. R. (1973) Biochim. Biophys. Acta 292, 130–139.
[13] Crofts, A. R., Wraight, C. A. and Fleischman, D. E. (1971) FEBS Letters 15, 89–100.
[14] Clayton, R. K. (1969) Biophys J. 9, 60–76.
[15] Tollin, G., Fujimori, E. and Calvin, M. (1958) Proc. Natl. Acad. Sci. U.S. 44, 1035–1047.
[16] Sweetser, P. B., Todd, C. W. and Hersh, R. T. (1961) Biochim. Biophys. Acta 51, 509–518.
[17] Jursinic, P. and Govindjee (1972) Photochem. Photobiol. 15, 331–348.
[18] Malkin, S. and Hardt, H. (1973) Biochim. Biophys. Acta 305, 292–301.
[19] Arnon, D. I. (1949) Plant Physiol. 24, 1–15.
[20] Mar, T. and Govindjee (1971) Biochim. Biophys. Acta 226, 200–203.
[21] Zankel, K. L. (1971) Biochim. Biophys. Acta 245, 373–385.
[22] Barbieri, G., Delosme, R. and Joliot, P. (1970) Photochem. Photobiol. 12, 197–206.