ON THE DETECTION OF A NEW RAPID RECOVERY KINETICS OF PHOTO-OXIDIZED CHLOROPHYLL-a_{II} IN ISOLATED CHLOROPLASTS UNDER REPETITIVE FLASH ILLUMINATION

G. RENGER, H.-J. ECKERT and H. E. BUCHWALD

Max Volmer Institut für Physikalische Chemie and Molekularbiologie der Technischen Universität, Strasse des 17. Juni 153, D-1000 Berlin 12, Germany

Received 22 March 1978

1. Introduction

The photosynthetic water cleavage into metabolically-bound hydrogen and molecular oxygen is initiated by the photochemical generation of sufficiently strong oxidizing equivalents within the reaction centers of system II. The primary reactions were shown to be accompanied by reversible photobleaching of a special chlorophyll-*a* complex, designated as chlorophyll-a_{II} (Chl-a_{II}) [1,2]. The bleaching has been ascribed to the photooxidation of Chl-a_{II}, whereas the recovery was assumed to reflect its subsequent dark reactions with secondary donors [3,4]. The reduction kinetics appears to be complex, with two clearly resolved components characterized by half-lives of 35 μs and 200 μs [1–4]. Different lines of indirect evidence have led to the suggestion, that there exists even faster kinetics within the reduction pathway of Chl-a_{II}^{+}, which are of the order of $\leqslant$1 μs [5–7]. Furthermore, the fast kinetics were inferred to appear only if the water-splitting enzyme system Y remains functionally intact [6,7]. The extent of this fast reduction is probably dependent on the charge accumulation state of system Y [6] and of the pH of the inner thylakoid space [8]. However, up to now there is no direct proof for the existence of the fast reduction kinetics of Chl-a_{II}^{+}. This paper provides measurements of highly-resolved absorption changes in the red region of Chl-a_{II}, which clearly demonstrate a fast μs recovery of photobleached Chl-a_{II}^{+}. As this reaction takes place only if the water-splitting enzyme system Y is operative, its appearance reflects the existence of an electron donor component, intimately connected with Chl-a_{II} and system Y, whose reactivity is drastically changed by the destruction of Y.

2. Materials and methods

Normal and Tris-washed chloroplasts were prepared from market spinach as in [9] and [10], respectively, as described in [11]. The reaction mixture contained: chloroplasts (5 μM chlorophyll), 0.3 mM $K_3[Fe(CN)_6]$, 10 mM KCl, 2 mM $MgCl_2$ and 20 mM *N*-tris(hydroxymethyl)methylglycine (tricine)–NaOH at pH 7.5 or 20 mM morpholinoethanesulfonate (MES)–NaOH at pH 6.0. The assay conditions for the oxygen measurements were the same except for a 10-fold higher chlorophyll concentration.

The absorption changes were recorded by a repetitive flash photometer modified by the application of 14 MHz-modulated detecting light [12] in order to eliminate fluorescence artefacts due to the actinic flashes. In order to improve the signal/noise ratio 8192 signals were averaged per measurement in a Biomation type 8100 and NIC LAB 80, but the sample was changed after each 1024 flashes.

Photosynthesis was excited with ultra-short flashes (PEK XE 9-3) of a duration of 1 μs at a repetition rate of 2 Hz. The flash light passed a Schott filter BG 23/5. The optical pathlength was 20 mm, the intensity of the monitoring light $\leqslant$ 30 μW/cm^2, optical bandwidth 10 nm. In order to eliminate fast kinetics due to the photosystem I turnover [13] far-

red background illumination ($\lambda = 722$ nm, $\Delta\lambda = 15$ nm) has been applied with an intensity of 290 $\mu W/cm^2$. Oxygen measurements were performed as in [14].

3. Results

The time course of the ΔA_{690} in isolated spinach chloroplasts at pH 7.5 is shown in fig.1, top. Despite the elimination of the Chl-a_I recovery in the μs-range by far-red illumination the absorption changes still contain a small Chl-a_I contribution with a half-life of about 20 ms, which appears as a constant bleaching on the 100 μs time scale. Accordingly, for a kinetic analysis this contribution has to be subtracted. It amounts about 15% in normal chloroplasts under saturating flash excitation. If one supposes that for non-saturating flashes nearly the same pattern arises, the contribution indicated by the broken line in fig.1, top, is obtained. The signals corrected for the Chl-a_I effect is depicted as a semilogarithmic plot in fig.1, bottom. It is clearly seen that in normal chloroplasts the recovery of Chl-a_{II} is characterized by triphasic kinetics. As in normal chloroplasts the 200 μs phase has a comparatively small amplitude and because of the limited accuracy of the measurements an exact separation is impossible. Therefore, these kinetics are indicated by a broken line. The remaining part, however, cannot be described simply by a single exponential decay, but it is rather seen to contain 2 different kinetics. The slower phase corresponds with the 35 μs kinetics [3,4,6,8]. Additionally, an as yet unknown, even faster, phase is clearly resolved with a half life of less than 5 μs. We tentatively assign this rapid decay to the 1 μs kinetics theoretically derived on the basis of earlier measurements [5–8]. It must be emphasized that in a series of measurements the half-life of the fast kinetics slightly varied in the range 2–4 μs. However, despite these deviations, in all experiments with normal chloroplasts the rapid μs decay was found. Similar results were obtained at pH 6.0, but the extent of the 35 μs kinetics is greater than that at pH 7.5. These data are in qualitative correspondence with [8].

In order to show that the very fast μs kinetics (vide supra) reflects the Chl-a_{II} turnover, the difference spectrum has been measured for the red region in the range of 670–710 nm. The experimental data depicted in fig.2 show that the difference spectrum in

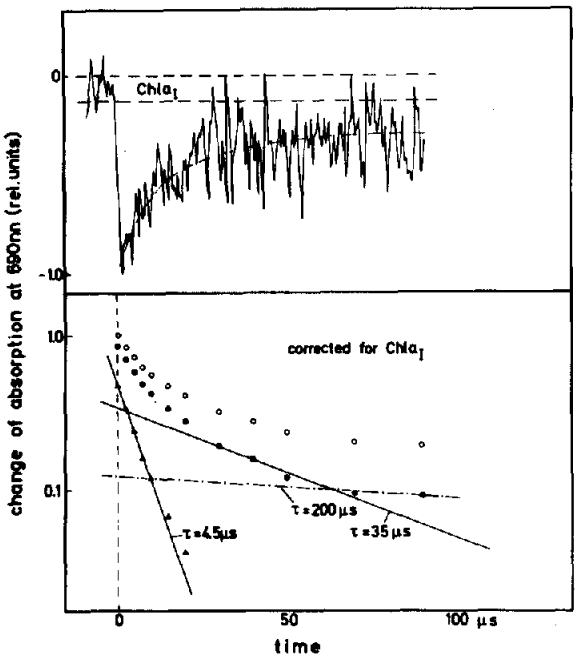

Fig.1. ΔA_{690} as a function of time in isolated spinach chloroplasts at pH 7.5. At t 0 a short non-saturating flash was fired. Top: linear plot. Bottom: Semilogarithmic plot of the absorption change (corrected for the contribution due to Chl-a_I) involving the separation of different kinetics.

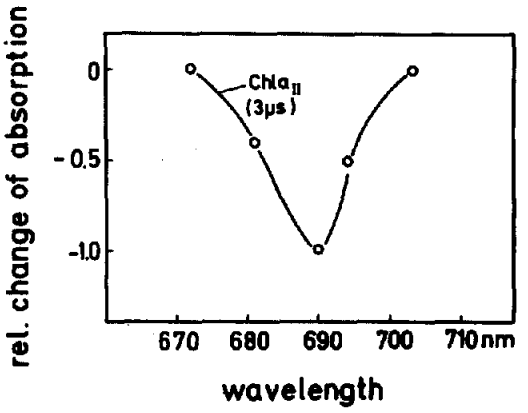

Fig.2. Transient difference spectrum of the very fast μs-kinetics (symbolized as 3 μs-component) induced by short non-saturating flashes in isolated spinach chloroplasts. The spectrum is normalized to the amplitude obtained at 690 nm.

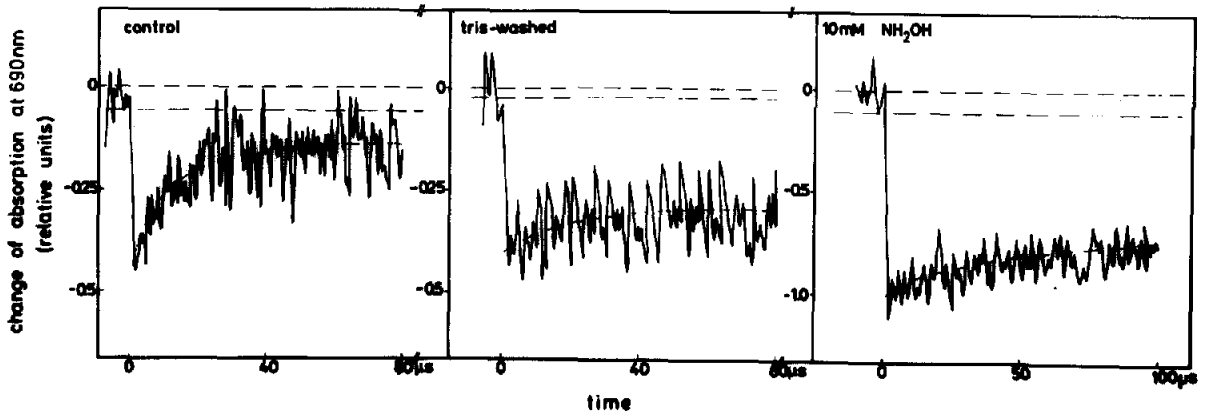

Fig.3. ΔA_{690} as a function of time in normal, Tris-washed and hydroxylamine-treated chloroplasts, normalized to the amplitude of NH_2OH-treated chloroplasts.

the red of the very fast μs kinetics (designated as 3 μs component) has a pronounced peak around 690 nm. The spectrum closely resembles that in [3] for the sum of the 35 μs and 200 μs kinetics, respectively. Therefore, the very fast μs kinetics belong most probably to the same species, i.e., the photooxidized Chl-a^+_{II} becoming reduced by secondary electron donors. The rapid kinetics of Chl-a^+_{II} reduction have been claimed to be intimately related to the functional integrity of the water-splitting enzyme system Y [5,6,15]. Therefore, the very fast μs-recovery kinetics are anticipated to disappear in mistreated chloroplasts, which are unable to evolve oxygen, but remain intact with respect to the primary charge separation at photosystem II. Chloroplasts of this type are obtainable by several treatments, such as Tris-washing [10,16] or incubation with hydroxylamine (NH_2OH) [5,17,18]. In fig.3 the ΔA_{690} in Tris-washed and hydroxylamine-treated chloroplasts are compared with that of normal chloroplasts. It is clearly demonstrated that after the destruction of the water-splitting enzyme system Y by Tris-washing or incubation with 10 mM NH_2OH the very fast μs kinetics are not observable, whereas at NH_2OH concentrations (10 μM) not sufficiently high for the damage of system Y [19] confirmed by O_2 measurements this rapid reaction still occurs (data not shown). These results indicate, that the very fast μs kinetics are closely related to the functional connection between Chl-a_{II} and the intact system Y. In chloroplasts treated with high NH_2OH concentrations the amplitude of the ΔA_{690} is nearly doubled. If one accepts that NH_2OH does not seriously change the saturation curve or the optical properties of Chl-a_{II}, the amplitude increase might be explained by the assumption of further even much faster kinetics existing in normal chloroplasts (vide infra), which is slowed down by NH_2OH. However, as NH_2OH is able to act as a donor for system I too, no unambigious conclusion can be drawn.

4. Discussion

The results presented in this paper confirm earlier theoretical suggestions, that in intact chloroplasts there exists under steady state excitation conditions a rapid reduction of photooxidized Chl-a^+_{II}, much faster than the previously described kinetics with half lives of 35 μs [3,4,6,8] and 200 μs [1,2], respectively. The half life of the very fast reduction kinetics has been found to vary from 2–5 μs. This time is slightly greater than that proposed earlier theoretically on the basis of indirect evidence. It must be emphasized that due to the limited accuracy of the measurements only a rough estimation can be made. However, a theoretical analysis (H.-J. E. and G. R., unpublished) leads to the conclusion that the half life of the very fast recovery kinetics of Chl-a_{II} described here cannot be significantly shorter than 1 μs. On the other hand, we cannot exclude the existence of additional faster recovery kinetics in the ns range, which would escape our detection. A first hint for the existence of a much

faster sub-μs reaction in photosystem II is the discovery of the 25 ns fluorescence increase after excitation of dark-adapted algae with a 2 ns laser flash [20]. However, it is very important to stress that the 25 ns kinetics do not occur in algae pre-illuminated with single turnover flashes. Accordingly, the 25 ns rise was ascribed to a singular priming reaction at photosystem II in the completely dark-adapted state. Furthermore, the emission increment due to the 25 ns rise is structureless in the range 640–720 nm lacking the typical red peak around 685 nm of chlorophyll-*a* fluorescence.

After completion of this study we became aware of the data [21], where a 30 ns relaxation phase of the laser flash-induced ΔA_{820} was found, which is supposed to be due to Chl-a_{II}^{+}. Unfortunately, the measurements were performed with dark-adapted chloroplasts only, so that the physiological role under natural illumination conditions beyond a priming reaction remains obscure.

The measurements of the present study were made under flash excitation conditions assuring the normal steady state of activation of the water-splitting enzyme system Y. Furthermore, in order to avoid any interference with double hit effects [6,22] the chloroplasts were excited with non-saturating flashes simultaneously excluding carotenoid triplet formation [23] (vide infra). The present data lead to the conclusion, that under normal environmental illumination conditions Chl-a_{II}^{+} becomes reduced by at least a triphasic kinetic pattern, with half-lives of $\leqslant 5$ μs, 35 μs and 200 μs, respectively. A comparison with the data found in NH_2OH-treated chloroplasts might suggest the existence of an even much faster phase, whose appearance and functional role under normal illumination conditions still remains unresolved. At pH 7.5 the overall reduction very likely occurs predominantly via very fast kinetics, in agreement with recent conclusions [8,15]. The details of Chl-a_{II}^{+} reduction pattern as a function of the physiological conditions (internal pH, charge accumulation state of system Y [6,8]) remain to be clarified. It should be mentioned that 3 μs kinetics have been detected also in pre-illuminated samples for the fluorescence rise [5,20]. However as the 3 μs rise might be caused by Chl-a_{II}^{+} reduction as well as by the decay of another quencher (probably a carotenoid triplet) produced by the flashes, no unequivocal conclusions can be drawn on the basis of these data.

The very fast μs kinetics of the Chl-a_{II} recovery was shown to disapper after the destruction of the water-splitting enzyme system Y (fig.3). As the reversal of Chl-a_{II}^{+} bleaching is ascribed to the reduction of photooxidized Chl-a_{II}^{+}, the present results indicate that an electron donor D_1 responsible for the rapid reduction kinetics becomes functionally blocked by the degradation of system Y. For the substantiation of D_1 as well as for the mechanism of its modification further investigations are required.

Acknowledgements

The authors are very grateful to Professor Dr H. T. Witt for his stimulatory interest in this work. They are thankful to Dr P. Mathis for a preprint of [21]. We also wish to thank Mrs R. Hachtmann for technical assistance and Mrs A. Schulze for drawing the figures. The financial support of ERP-Sondervermögen, by the Commission of European Communities and of Deutsche Forschungsgemeinschaft is gratefully acknowledged.

References

[1] Döring, G., Stiehl, H. H. and Witt, H. T. (1967) Z. Naturforsch. 22b, 639–644.
[2] Döring, G., Renger, G., Vater, J. and Witt, H. T. (1969) Z. Naturforsch 24b, 1139–1143.
[3] Gläser, M., Wolff, Ch., Buchwald, H. E. and Witt, H. T. (1974) FEBS Lett. 42, 81–85.
[4] Wolff, Ch., Gläser, M. and Witt, H. T. (1975) Proc. 3rd. Inf. Congr. Photosynthesis (Avron, M. ed) pp. 295–305, Elsevier, Amsterdam, New York.
[5] Duysens, L. M. N., Den Haan, G. A. and Van Best, J. (1975) Proc. 3rd Int. Congr. Photosynthesis (Avron, M. ed) pp. 1–12, Elsevier, Amsterdam, New York.
[6] Gläser, M., Wolff, Ch. and Renger, G. (1976) Z. Naturforsch. 31c, 712–721.
[7] Van Best, J. and Duysens, L. M. N. (1977) Biochim. Biophys. Acta 459, 187–206.
[8] Renger, G., Gläser, M. and Buchwald, H. E. (1977) Biochim. Biophys. Acta 461, 392–402.
[9] Winget, G. D., Izawa, S. and Good, N. E. (1965) Biochem. Biophys. Res. Commun. 21, 438–443.
[10] Yamashita, T. and Butler, W. L. (1968) Plant Physiol. 43, 1978–1984.
[11] Erixon, K. and Renger, G. (1974) Biochim. Biophys. Acta 333, 95–106.
[12] Buchwald, H. E. (1968) Thesis, Technical University Berlin.

[13] Haehnel, W., Döring, G. and Witt, H. T. (1971) Z. Naturforsch. 26b, 1171–1174.
[14] Renger, G. (1972) Biochim. Biophys. Acta 256, 428–439.
[15] Mathis, P., Haveman, J. and Yates, M. (1976) Brookhaven Symp. Biol. 28, 267–277.
[16] Renger, G. and Wolff, Ch. (1976) Biochim. Biophys. Acta 423, 610–614.
[17] Cheniae, G. M. and Martin, I. F. (1970) Biochim. Biophys. Acta 197, 219–239.
[18] Mathis, P. and Van Best, J. A. (1978) Proc. 4th Int. Congr. Photosynthesis (Hall, D. O., Coombs, J. and Goodwin, T. W. eds) pp. 387–396, The Biochemical Society, London.
[19] Bouges, B. (1971) Biochim. Biophys. Acta 234, 103–112.
[20] Mauzerall, D. (1972) Proc. Natl. Acad. Sci. USA 69, 1358–1362.
[21] Van Best, J. A. and Mathis, P. (1978) Biochim. Biophys. Acta 503, 178–188.
[22] Joliot, P. and Joliot, A. (1977) Biochim. Biophys. Acta 462, 559–574.
[23] Wolff, Ch. and Witt, H. T. (1969) Z. Naturforsch. 24b, 1031–1037.