STUDIES ON THE KINETICS OF THE 515 nm ABSORBANCE CHANGES IN CHLOROPLASTS

Evidence for the induction of a fast and a slow P515 response upon saturating light flashes

A. H. C. M. SCHAPENDONK, W. J. VREDENBERG and W. J. M. TONK

Centre for Agrobiological Research, PO Box 14, 6700 AA Wageningen, The Netherlands

Received 8 February 1979

1. Introduction

A great deal of attention has been given in recent years to the functional relationship between the generation of an electrical potential, proton transfer, conformational changes and ATP synthesis in chloroplast membranes, associated with the primary photosynthetic light reactions [1–7]. Conclusions about magnitude and time course of the transmembrane potential were obtained either by means of indirect potential measurements such as the light-induced ΔA_{515} of the carotenoid chlorophyll *b* pigment complex (P515), or by direct measurements of the chloroplast (thylakoid) transmembrane potential with micro-capillary glass electrodes [3,8–11]. The rise and decay of the field-indicating ΔA_{515} upon single turnover saturating light flashes have been reported to occur with single first-order reaction kinetics in broken chloroplasts suspended in isotonic medium under non-phosphorylating conditions [1,12,13]. The rate constant of the dark decay appears to be determined by the passive ion permeability of the membrane. The dark decay has been shown to become biphasic in the presence of ADP and inorganic phosphates. This has been suggested to be due to potential-dependent changes in the proton conductance through the ATP synthesizing enzyme complex [14–18]. Recently it has been reported that the flash-induced P515 ΔA in intact (class I) chloroplasts, like in intact algal cells [19], occurs with complex multi-phasic rise and decay kinetics [20]. However, rise and decay of the flash-induced potential changes in intact chloroplasts measured with micro-electrodes have been shown to occur with single first-order reaction kinetics [21–23]. The rate constant of the potential change after a single flash was found to be dependent on the ionic permeability of the thylakoid membrane [23]. Apparent discrepancies on the magnitude and kinetics of the light-induced transmembrane potential (changes), as concluded from P515 and micro-electrode measurements have been long a matter of discussion [3,8–10,24]. This communication deals with analyses of the kinetics of flash-induced ΔA of P515 and of the transmembrane potential, studied on thylakoid membranes of dark-adapted spinach and *Peperomia* chloroplasts, respectively. The analyses suggest evidence that the ΔA_{515} in intact and broken chloroplasts is the composite result of at least two different processes, which are called reaction I and reaction II. The kinetics of the ΔA caused by reaction I, characterized by a rise time of < 0.5 ms and a decay rate of 8.6–17.3 s^{-1}, show similarities with the kinetics of the transmembrane potential, measured by means of micro-capillary glass electrodes. The ΔA associated with reaction II, characterised by a rise time of 100–150 ms and a decay rate of 1.1–1.7 s^{-1}, is presumed to be a reflection of slow intramembranal structural changes which are induced by field-dependent charge displacements in the vicinity of the P515 pigment complex.

2. Materials and methods

Intact and broken chloroplasts were isolated from fresh grown spinach leaves and prepared by the method in [25], except for the following modifications with respect to the incubation and assay medium. Intact chloroplasts were incubated and measured in a medium

which contained 330 mM sorbitol, 0.5 mM K_2HPO_4, 10 mM $NaHCO_3$, 2 mM Hepes (pH 7.5) and 10 mM $MgCl_2$. Broken chloroplasts were obtained by osmotic shock in H_2O containing 10 mM $MgCl_2$ or 10 mM KCl. The assay medium contained 330 mM sorbitol, 30 mM tricine (pH 7.7) and 10 mM $MgCl_2$. Chloroplast concentration usually was equivalent to ~50 μg chl.ml^{-1}. Preparations were preincubated in the dark for 1 h; preincubation and measurements occurred at 2°C.

Absorbance changes at 515 nm, induced by single turnover flashes (half-life 8 μs, wavelengths > 665 nm), were measured with the set-up described in [25]. Either 1 flash or a group of 2–6 flashes with time intervals of 100 ms was fired. The repetition rate usually was 0.05 s^{-1}, the repetition number 32 or 64. In some experiments, notably those in which the response associated with reaction I was measured, 38 or 70 flashes were fired at a repetition rate of 4 s^{-1}. In this case the first 6 flashes were used for pre-conditioning the sample, i.e., for saturating reaction II. Sampling and averaging occurred by a DL 102A signal averager.

Flash-induced electric potential changes were measured on single chloroplasts in intact leaves of *Peperomia metallica*. Experimental conditions and methods were the same as in [10,22]. Potential changes were measured by means of micro-capillary glass electrodes inserted into a single chloroplast. Tip diameter of the electrode was < 0.2 μm. Flash illumination occurred by saturating single turnover flashes. Time resolution of the potential measuring system was ~0.1 ms.

3. Results and interpretation

A representative example of the time course of the ΔA_{515} upon a single turnover saturating light flash in intact chloroplasts is shown in fig.1a,b. Analyses of the semi-logarithmic plots of these changes reveal at least 5 different phases in the kinetics. After the initial fast absorbance increase, called phase a and completed within a time shorter than the resolution time of the measuring system (0.5 ms), a relatively slow increase in absorbance occurs in the first 10–20 ms up to an absorbance level which is more or less constant for the next 20–80 ms. According to the analysis, the

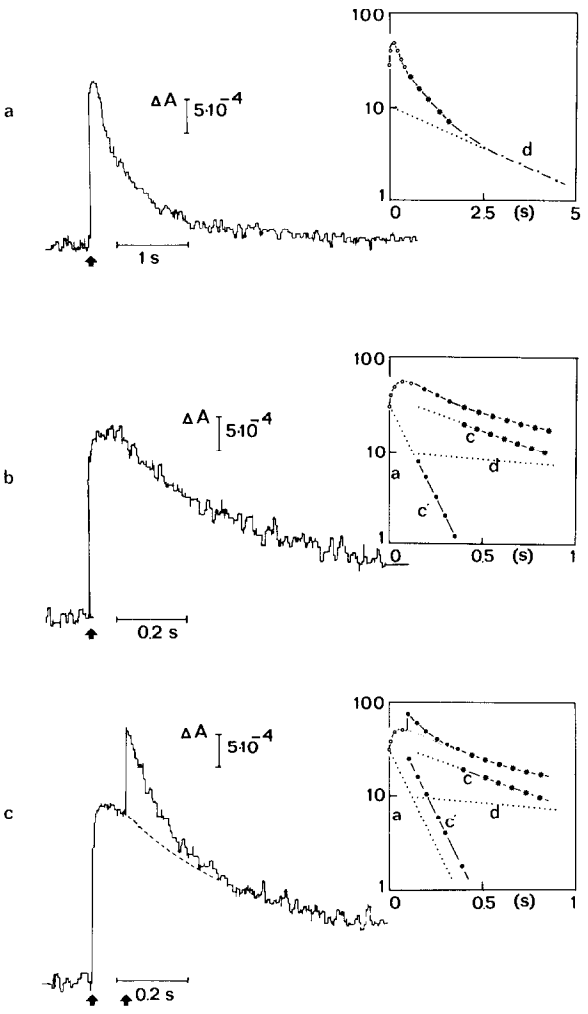

Fig.1. Absorbance changes at 515 nm in dark-adapted intact chloroplasts, induced by single (a,b) or double (c) flashes, recorded and displayed on a 5 s (a) and 1 s time scale (b,c). Average of 32 single or double flashes, fired at a rate of 0.05 s^{-1}. The arrows mark the moments at which the flashes were fired. Inserts: Semi-logarithmic plots of the experimental curves and the curve analyses into exponential phase d (open squares), c (closed stars) and c′ (closed circles), respectively, according to an adopted method (c.f. [32]). Extrapolation of phase d and subtraction of the absorbance of phase d from the experimental absorbance at corresponding times, gives the ΔA in the absence of phase d. This absorbance curve yields the measuring points of phase c and, after repeating the same procedure, those of phase c′. The dotted line parallel with phase c′ (lower right-hand figure) represents the fast phase c′ induced by the first flash (middle right-hand figure). Other dotted lines represent the extrapolated part of the respective phases.

subsequent decrease in absorbance appears to be composed of three different phases. A major phase c with a half-life of 420 ms is preceded by a relatively fast phase c′ with a half-life of 75 ms, and followed by a small slow phase d decaying with a half-life of 1.5 s. Phases c′, c and d are not defined during the first 70–100 ms after the flash due to the occurrence of the slow absorbance rise or even constant level in this time domain. However, an extrapolation of the decay line of phase c′ to time zero (fig.1b) would result in an initial level which coincides reasonably well with the one reached in the fast phase a rise. This certainly is not true for phase c. The data of fig.1b suggest that the phase c′ decay is associated with the phase a rise. This suggestion is substantiated by the analysed data of the absorbance response in a double flash (fig.1c). These show that the ΔA upon a second flash, characterised by a fast rise and a single decay phase equal to the c′ component analysed in the first flash, is superimposed upon the ΔA induced by the first flash. The break between the fast (c′) and the slow (c) phase after the second flash is at an absorbance level which is determined by the (phase c, d) ΔA caused by the first flash. The kinetics of the response upon third and following flashes, fired at time intervals in the range of 10–100 ms, were found to be similar to those induced by the second flash. Thus we arrive at the conclusion that the rapid decay component (phase c′) is associated with a fast rise (phase a). These components have been ascribed to a reaction called reaction I. The time course of reaction I is shown in fig.2. The

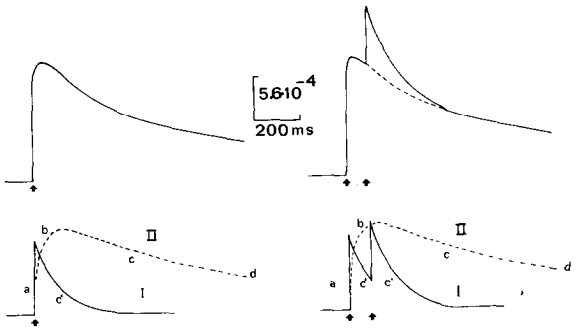

Fig.2. Resolution (bottom part of the figure), of the ΔA induced by a single and a double flash into two components; reaction I (solid curve) and reaction II (broken curve). Curves I and II were computed according to the data obtained from the curve analysis depicted in fig.1. Further explanations are in the text.

response obtained by subtracting reaction I from the overall P515 response has been attributed to that of reaction II (fig.2). The rise kinetics of reaction II show a slow absorbance increase within 140 ms. This is called phase b. The decay of reaction II after the flash is bi-phasic with rate constants determined by the characteristic relaxation times of phases c and d. For a large variety of preparations, the half-life of phase c′, c and d was found to be in the range from 40–80 ms, 400–600 ms and 1.5–3 s, respectively. Figure 2, representative for intact chloroplasts, shows that the A_{515} increases associated with reaction I and II (phase a and b, respectively) are about equal in magnitude. The spectra of these changes were found to be identical to the characteristic difference spectrum of P515 [25]. This leads to the suggestion that the ΔA associated with reaction I and II are due to an electrochromic response of the P515 pigment complex. The spectrum of the relatively small phase d was found to be partially dissimilar with that of P515. Phase d, which apparently is negligibly small in broken chloroplasts (see below) might represent the decay of a reaction different from reaction I and II. Therefore reaction II in intact chloroplasts might need a small correction for the ΔA associated with phase d. According to the data of fig.1,2 one would expect for intact chloroplasts that, after one pre-illuminating flash, single flashes fired at a repetition rate of 4–5 s^{-1} cause the P515 response of reaction I, i.e., an ΔA with a fast rise (phase a) and a single exponential decay (phase c′) with a rate constant of ~9.2 s^{-1}. It should be emphasized that this response is superimposed upon an (constant) absorbance level determined by the ΔA induced in the pre-illumination flash by reaction II. Experiments of this kind (not shown for intact chloroplasts, but see fig.3) have nicely confirmed our conclusion.

Figure 3 illustrates that the P515 response in broken chloroplasts also is composed of a reaction I- and reaction II-type. Figure 3a shows the response of P515 in a fresh preparation of dark-adapted broken chloroplasts, suspended in a Mg-containing medium, illuminated by flashes fired at a repetition of 0.05 s^{-1}. The semi-logarithmic plot clearly shows that the decay is biphasic with a slow decaying component (phase c, rate constant ~1.4 s^{-1}). After pre-illumination with 4–6 flashes, flashes fired at a repetition rate of 4 s^{-1} cause a response (fig.3b) which exhibits a single exponential decay (phase c′) with a rate constant of ~12 s^{-1}.

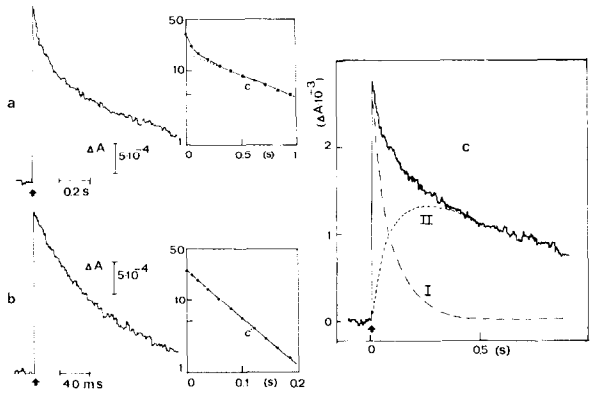

Fig.3. Time courses of the ΔA_{515} in broken chloroplasts (in the presence of 10 mM $MgCl_2$), induced by repetitive single flashes fired at a repetition rate of 0.05 s^{-1} (a, c) and 4 s^{-1} (b). Curve b was measured immediately after preillumination with 6 flashes, fired at a rate of 4 s^{-1}. Inserts in a and b: Semi-logarithmic plot of the absorbance change against time. Note the different time scales in a and b. The dashed curve (I) in c is identical to the response measured in b, i.e. characterized by an exponential decay phase c'; curve II is the difference between the experimental curve (identical to the one shown in a) and curve I. Further explanations are in the text.

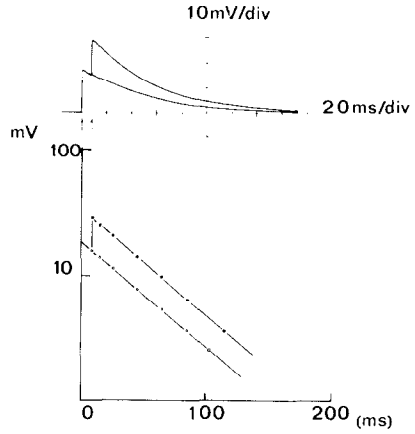

Fig.4. Response and semi-logarithmic plot of the photoelectric potential of chloroplast (thylakoid) membranes to a saturating light flash or to a series of two saturating light flashes separated by a dark time of 20 ms. Measurements were done in a cross section of a *Peperomia* leaf with a microcapillary glass electrode inserted into a single chloroplast.

Because of the plain similarities with the occurrences in intact chloroplasts, this response is attributed to reaction I. Subtraction then gives the response of reaction II, as illustrated in fig.3c (dashed curve). The increase in absorbance caused by reaction II is completed in ~250 ms and amounts to ~50% of the increase caused by reaction I. For the experiment shown here the saturation level of the ΔA associated with reaction II was found to be reached after 4–6 flashes, fired at an interval of 200 ms. It was about twice as high as the change after one flash.

Figure 4 shows the single and double flash response of the potential across the chloroplast (thylakoid) membrane of a *Peperomia* chloroplast in situ measured with a micro-electrode. The analyses indicate a single and equal exponential potential decay after the first and second flash with a half-life of 35 ms. In a few instances the relaxation after the second flash was found to be slightly (~10%) faster than after the first flash. The half-life of the potential decay for a large variety of different chloroplasts was found to be 30–60 ms. The potential increase measured in the first flash was found to be in the 15–40 mV range; the ratio between the potential change in the second and first flash was 0.6–0.8.

Because of plain similarities between the kinetics of reaction I (fig.2) and the kinetics of the transmembrane potential changes as measured with micro-capillary glass electrodes (fig.4) there is reasonably good evidence for the conclusion that the ΔA_{515} associated with reaction I is due to the transmembrane electric field. It seems unlikely that the slow decay kinetics of reaction II, which are mainly, if not exclusively, determining the decay of the overall P515 response after a flash in the 0.1–1 s time domain (c.f. fig.1,2) are associated with the decay of the transmembrane electric field.

It has been reported [23] that the decay of the transmembrane potential in the presence of valinomycin is enhanced by a factor of ~5. However valinomycin was found to cause an enhancement of the overall P515 decay after a single flash by a factor of 500–1000 (A.H.C.M.S., W.J., in preparation). According to analyses, similar to those shown in fig.1,3, it has been observed that this disproportional acceleration of the P515 overall decay is due to a suppression of reaction II. In general, the magnitude of reaction II in broken chloroplasts, unlike that of reaction I was found to be strongly dependent on the physiological

condition of the chloroplasts and to be substantially suppressed by membrane modifying substances, divalent cations and after ageing and freezing. The kinetics and spectral characteristics of reaction II suggest that its manifestation is a reflection of physicochemical processes occurring in the vicinity of the P515 pigment complex in the intramembrane phase. We propose that the electric field associated with the primary charge separation in the reaction centers, induces conformational changes in the structural assembly of lipid and protein molecules in the membrane core. These changes are supposed to alter the mutual orientation of fixed charged groups of the molecular structures within the membrane. This is suggested to result in (a change in) the exposure of pigments of the field-sensitive P515 complex to the intrinsic electric field associated with these charge displacements. This exposure as a consequence will result in ΔA_{515} of the reaction II type. It is interesting to note that the rise and decay kinetics of the P515 absorbance change associated with reaction II closely resemble the optical response of the potential probe oxonol VI [26,27]. In general it appears that the rise of reaction II occurs in the same time range as the flash-induced response of external probes which are indicators of either changes in the membrane surface potential [28], or of conformational changes of the chloroplast ATPase [29]. Further studies are needed to establish the origin and mechanism of reaction II. It might be that the ΔA_{515} associated with reaction II is a useful intrinsic marker of the conformation and energy coupling capacity of the thylakoid membrane. It has been discussed [30,31] that some mode of membrane energization or conformation is needed for the onset of ATP synthesis in the light. The relaxation time which has been determined for this activation step appears to be in the same time range as that of the rise of reaction II.

Acknowledgements

This research was partly supported by the Netherlands Foundation for Biophysics (Stichting voor Biophysica), financed by the Netherlands Organization for the Advancement of Pure Research (ZWO). The authors are grateful to Dr R. Kraayenhof and J. J. Schuurmans for valuable discussion.

References

[1] Witt, H. T. (1971) Quart. Rev. Biophys. 4, 365–477.
[2] Junge, W. (1977) in: Encyclopedia of Plantphysiology, new ser. (Trebst, A. and Avron, M. eds) vol. 5, pp. 59–93, Springer-Verlag, Berlin.
[3] Junge, W. (1977) Ann. Rev. Plant Physiol. 28, 503–536.
[4] Jagendorf, A. T. (1977) in: Encyclopedia of Plantphysiology, new ser. (Trebst, A. and Avron, M. eds) vol. 5, pp. 307–337, Springer-Verlag, Berlin.
[5] Avron, M. (1977) Ann. Rev. Biochem. 46, 143–155.
[6] Reeves, S. G. and Hall, D. O. (1977) Biochim. Biophys. Acta 463, 275–297.
[7] Robertson, R. N. and Boardman, N. K. (1975) FEBS Lett. 60, 1–6.
[8] Rumberg, B. (1977) in: Encyclopedia of Plantphysiology, new ser. (Trebst, A. and Avron, M. eds) vol. 5, pp. 405–415, Springer-Verlag, Berlin.
[9] Vredenberg, W. J. (1976) in: The Intact Chloroplast (Barber, J. ed) Topics Photosyn. vol. 1, pp. 53–88, Elsevier, Amsterdam, New York.
[10] Vredenberg, W. J. and Tonk, W. J. M. (1975) Biochim. Biophys. Acta 387, 580–587.
[11] Gräber, P. and Witt, H. T. (1975) Biochim. Biophys. Acta 423, 141–163.
[12] Junge, W. and Witt, H. T. (1968) Z. Naturforsch. 23b, 244–254.
[13] Boeck, M. and Witt, H. T. (1972) in: Proc. IInd Int. Congr. Photosyn. (Forti, G. et al. eds) vol. 2, pp. 903–911, W. Junk, The Hague.
[14] Schliephake, W., Junge, W. and Witt, H. T. (1968) Z. Naturforsch. 23b, 1571–1578.
[15] Rumberg, B. and Siggel, U. (1968) Z. Naturforsch. 23b, 239–244.
[16] Junge, W., Rumberg, B. and Schröder, H. (1970) Eur. J. Biochem. 14, 575–581.
[17] Schmid, R., Shavit, R. and Junge, W. (1976) Biochim. Biophys. Acta 430, 145–153.
[18] Girault, G. and Galmiche, J. (1978) Biochim. Biophys. Acta 502, 430–444.
[19] Joliot, P. and Delosme, R. (1974) Biochim. Biophys. Acta 357, 267–284.
[20] Horváth, G., Droppa, M., Mustárdy, L. and Faludi-Daniel, A. (1978) Planta 141, 239–244.
[21] Bulychev, A. A., Andrianov, V. K., Kurella, G. A. and Litvin, F. F. (1972) Nature 236, 175–176.
[22] Vredenberg, W. J. and Tonk, W. J. M. (1974) FEBS Lett. 42, 236–239.
[23] Bulychev, A. A. and Vredenberg, W. J. (1975) Biochim. Biophys. Acta 423, 548–556.
[24] Schapendonk, A. H. C. M. and Vredenberg, W. J. (1977) Biochim. Biophys. Acta 462, 613–621.
[25] Vredenberg, W. J. and Schapendonk, A. H. C. M. (1978) FEBS Lett. 91, 90–93.
[26] Schuurmans, J. J., Casey, R. P. and Kraayenhof, R. (1978) FEBS Lett. 94, 405–409.
[27] Bashford, L. C., Chance, B. and Prince, R. C. (1978) Biochim. Biophys. Acta 545, 46–57.

[28] Kraayenhof, R. and Arents, J. C. (1977) in: Electrical Phenomena at the Biological Membrane Level (Roux, E. ed) pp. 493–504, Elsevier, Amsterdam.
[29] Kraayenhof, R. and Slater, E. C. (1974) in: Proc. 3rd Int. Congr. Photosyn. (Avron, M. ed) pp. 985–986, Elsevier, Amsterdam.
[30] Ort, D. R., Dilley, R. A. and Good, N. E. (1976) Biochim. Biophys. Acta 449, 108–124.
[31] Vinkler, C., Avron, M. and Boyer, P. D. (1978) FEBS Lett. 96, 129–134.
[32] Atkins, G. L. (1969) in: Multicompartment Models for Biological Systems, p. 98, Methuen, London.