TRANSMEMBRANE ELECTRICAL POTENTIAL FORMATION IN SPINACH CHLOROPLASTS

Investigation using a rapidly-responding extrinsic probe

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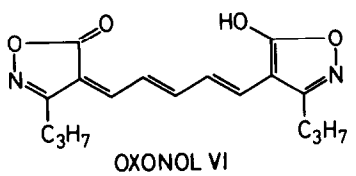
1. Introduction

It is well established that an electrochemical proton gradient ($\Delta\tilde{\mu}_{H^+}$) can drive ATP synthesis [1–3] and that a $\Delta\tilde{\mu}_{H^+}$ is formed during photosynthetic phosphorylation [4], consistent with the chemiosmotic hypothesis of Mitchell [5,6]. Whilst, in the steady state, the bulk of this $\Delta\tilde{\mu}_{H^+}$ is in the form of a ΔpH [7,8] and less in the form of its electrical component ($\Delta\psi$), considerable evidence indicates that there is a 'burst' of $\Delta\psi$ on illumination of chloroplasts, which decays rapidly to its steady state level [4,9]. Furthermore this initial $\Delta\psi$ appears to play a fundamental role in the regulation of photosynthetic phosphorylation [10]. Investigations of the initial $\Delta\psi$ formation have employed chloroplasts activated by flashes of saturating light, which induce a single turnover of the photosynthetic apparatus [11]. The rapid formation and decay of $\Delta\psi$ in this system have been observed, in the main, using two techniques. A shift in the absorbance spectrum of the carotenoids in the chloroplast-thylakoid membrane occur on illumination [9] and this shift is $\Delta\psi$-linked [12,13]. Micro-electrodes, implanted in the giant chloroplasts of *Peperomia metallica*, also detect a light-induced $\Delta\psi$ [14]. There are considerable differences, however, between the magnitude and kinetics of formation of the $\Delta\psi$, as measured using these two techniques. Moreover, both methods have been criticised concerning their ability to measure accurately bulk $\Delta\psi$ in chloroplasts [4]. Clearly, it would be of great advantage to complement the results obtained using these methods with studies using extrinsic optical probes of membrane potential, such as have been used in many other bioenergetic systems [15]. Most of these probes, however, have a slow response time relative to the rapid time course of the single turnover experiments. Merocyanine follows action potentials occurring in 10 ms in the giant axon of squid [16], but its response to $\Delta\psi$ is much smaller and slower in chloroplasts [17]. This difficulty has prevented the application of these probes in the past. Recently, however, a class of rapidly-responding optical probes of potential – the oxonols – has been developed. Oxonol V (bis [3-phenyl-5-oxoisoxazol-4-yl] pentanethineoxonol) responds rapidly to light-induced $\Delta\psi$ formation in chromatophores of photosynthetic bacteria [18] and oxonol VI (bis [3-propyl-5-oxoisoxazol-4-yl] pentamethineoxonol, structure shown below) to energisation of sub-mitochondrial particles [19].



OXONOL VI

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We show here that in spinach thylakoids, oxonol VI undergoes a spectral shift on $\Delta\psi$ formation, induced via a gradient of K^+ activity or by illumination. Furthermore, the probe response is sufficiently rapid as to allow its use in single-turnover experiments. The magnitude and kinetics of the $\Delta\psi$ induced by flash illumination, as measured with oxonol VI, are compared to those which have been observed using the carotenoid-shift and micro-electrode techniques.

2. Materials and methods

Thylakoids were prepared as in [13]. Steady illumination was applied through a Corning 2030 filter. Flash-induced absorption changes were measured using a dual-beam spectrophotometer, built in this laboratory. The sample contained in a 1.6 ml cuvette, fitted with thermostatic temperature control and a stirrer, and individual flashes (4 μs half-width) were applied using a General Electric FT-230 flash tube. Subsequent changes in absorbance were stored and averaged using a digital, transient recorder (Datalab DL-922) and an averager (Datalab DL-4000). Accumulated scans were recorded using a Hewlett-Packard 2004B $X-Y$ plotter. Other dual-wavelength measurements were carried out using an Aminco DW-2a spectrophotometer. Changes in pH were measured using an Lot-141-M3-RK glass electrode, built by Ingold (Zürich) to our specifications. Experimental medium contained 330 mM sorbitol/2 mM $MgCl_2$/2 mM Hepes (pH 7.2).

As uncouplers were used S-13 (5-chloro-3-*tert*-butyl-2′-chloro-4′-nitro-salicylanilide), kindly provided by Dr P. Hamm, Monsanto Co. St. Louis, and SF-6847 (3,5-di-*tert*-butyl-4-hydroxy-bensylidenemalononitrile), a generous gift from Dr H. Terada, University of Tokyshima, Japan. Valinomycin was a gift from Dr W. C. Pettinga, Eli Lilly, Indianapolis. Other reagents were obtained commercially at analytical grade.

3. Results and discussion

When spinach thylakoids were illuminated in the presence of oxonol VI, they accumulated protons from the external medium with the time course shown in fig.1. Concomitantly with this H^+ uptake, changes occurred in the absorbance maximum of oxonol VI [19], relative to a reference wavelength of 603 nm. At the onset of illumination, there was a rapid decrease of ~1% in the A_{590} which decayed, concurrently with H^+ uptake. to a steady level at which it remained until illumination was terminated. In the absence of thylakoids, similar illumination had no effect on oxonol VI absorbance. In fig.2 is shown a spectrum of oxonol VI in the presence of illuminated thylakoids minus that obtained in the dark. This difference spectrum indicates that illumination in the presence of chloroplasts leads to a shift in the principal absorbance peak of oxonol VI. It can also be seen in fig.2 that this light-induced spectral shift is mimicked by the induction of a transmembrane

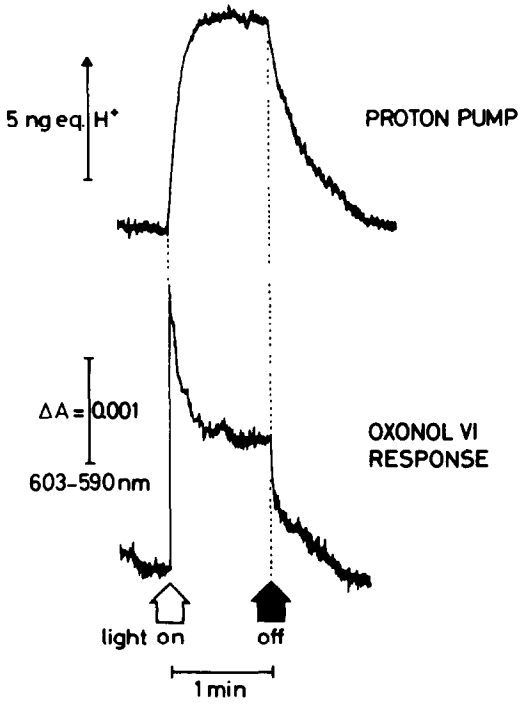

Fig.1. Proton uptake and oxonol VI absorbance changes in thylakoids under continuous illumination. Thylakoids were suspended in experimental medium containing 20 μM benzyl viologen to give 40 μg chl./ml at 20°C. Changes in the pH of the suspension occurring under steady illumination are shown in the upper trace. The lower trace shows the light-induced changes in the absorbance of 0.5 μM oxonol VI, present in the suspension under the same conditions.

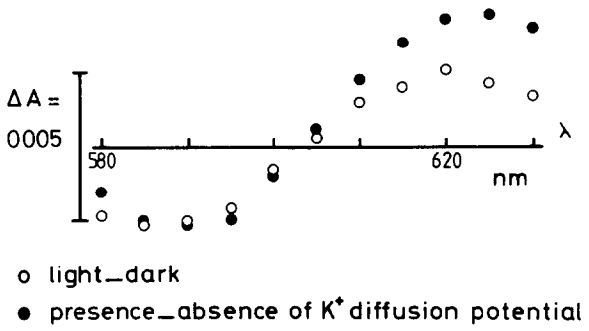

Fig.2. Energy-induced changes of oxonol VI spectrum. Thylakoids were suspended in the experimental medium in the presence of 0.5 μM oxonol VI to give 40 μg chl./ml at 20°C. The initial change in absorbance of the suspension occurring under steady illumination in the presence of 20 μM benzyl viologen (○) and the change in absorbance occurring in the presence of 3 μM valinomycin on the addition of 100 mM KCl (•) are shown as a function of wavelength of measured light.

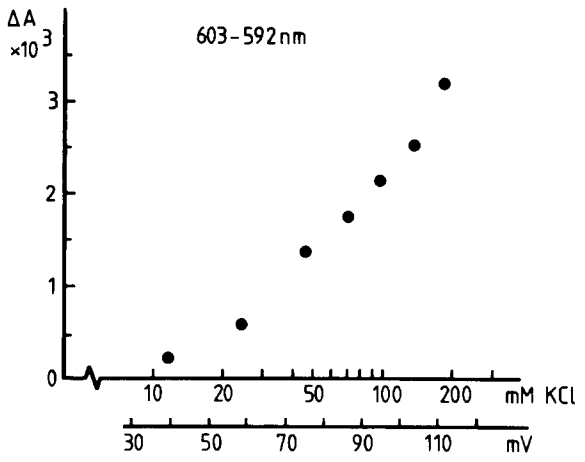

Fig.3. Dependence of oxonol VI ΔA_{590} in the presence of thylakoids and valinomycin on the applied K^+ concentration gradient. Thylakoids containing 1 mM KCl were prepared as in section 2, except that lysis was carried out in the presence of 1 mM KCl and the thylakoids were suspended in experimental medium containing 1 mM KCl. The thylakoid suspension was added to 2 ml experimental medium containing 1 mM KCl to give 40 μg chl./ml at 20°C. The Hepes buffer was adjusted to pH 7.2 with KOH, bringing the total K^+ to 2.3 mM in the experimental medium. This was followed by the addition of 6 μl 1 mg/ml valinomycin and 1 μl 1 mM oxonol VI. A mixture of 4 M KCl and 4 M NaCl, 100 μl, was then added to give the K^+ concentration indicated. The suspension was then stirred and the increase in $A_{603-590}$ was measured after 30 s when it had reached a steady level. The lower scale of the x-axis shows the $\Delta\psi$ as calculated from 58.2 $\log_{10}$ ([K^+] out/[K^+] in).

electrical potential difference via a K^+-activity gradient in the presence of valinomycin. The above observations imply that the rapid decrease in A_{590} occurring on illumination was a consequence of $\Delta\psi$ formation across the thylakoid membrane. The subsequent increase in A_{590}, concomitantly with H^+ translocation, may then be accounted for by a decay of the oxonol VI shift as the $\Delta\psi$ was dissipated, according to the scheme of light-induced ion movement proposed [9]. Further support for this suggestion is given by the observation that a decrease in the A_{590} of oxonol VI following flash illumination was abolished by valinomycin and K^+, and was decreased greatly by the uncoupler S-13 (fig.4). SF-6847 showed an identical effect on the oxonol VI response, but caused a transient increase in the A_{590}, following the decay of the oxonol response (not shown).

The change in A_{590} (relative to that at 603 nm) of oxonol VI added to a suspension of thylakoids, was measured as a function of the applied electrical potential gradient (fig.3). This was varied using different external KCl concentrations, in the presence of valinomycin whilst keeping the external ionic strength and osmolarity, and the internal K^+ concentration constant. The value of the calculated potentials must be viewed with some caution, as the activity coefficient of K^+ inside and outside the thylakoids may not be the same. Bearing in mind this reservation, the values of the initial and steady state electrical potentials shown in fig.1, are 90 mV and 50 mV, respectively.

The kinetics and decay of the Oxonol VI spectral shift following flash illumination were examined using the averaging technique described in section 2. These are shown in fig.4, in comparison to those of the carotenoid response observed under the same conditions. The onset of the oxonol VI shift had a half-time of ~20 ms. This is quite close to some of the response times reported for implanted micro-electrodes (e.g., 10–15 ms [21]). It is much slower, however, than that of the carotenoidshift onset, which was too rapid to be resolved by the instrumentation used here but has

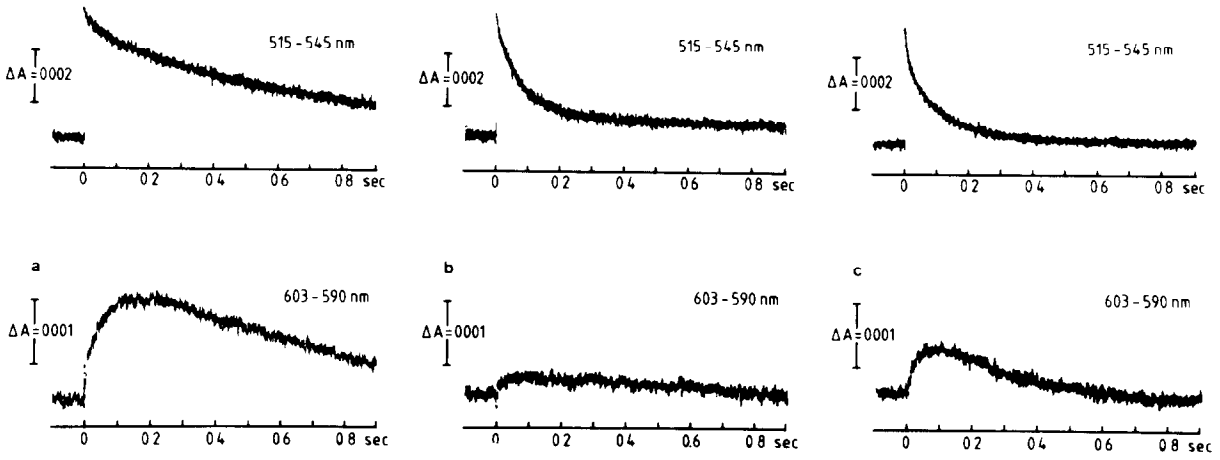

Fig.4. Flash-induced oxonol VI, and carotenoid, absorbance changes in the presence of thylakoids. (a) Thylakoids were suspended in 1.6 ml experimental medium containing 20 μM benzyl viologen at 20°C. Repetitive flash illumination of this sample was carried out, and subsequent absorbance changes were recorded as in section 2. In the upper trace are shown the flash-induced changes in $A_{515-545}$ (carotenoid absorbance), and in the lower trace, those in $A_{603-590}$ (oxonol VI absorbance). An interpulse time of 2 s was used and all flash-induced changes had decayed totally in this time. In the upper and lower traces of (b) and (c) are shown the changes in carotenoid and oxonol VI absorbance, respectively, occurring under the above conditions in the presence of 1 mM KCl and 0.5 μM valinomycin (b) or of 50 nM S-13 (c). The ionophores were added from ethanolic solutions. The ethanol thus introduced in the sample did not exceed 0.2% (v/v) and, at this concentration, did not effect the oxonol response.

been determined to be < 20 ns [9]. The onset of the oxonol VI shift also occurs in the same time-range ($t_{1/2}$ = 20–60 ms) as the light-induced change in the thylakoid surface potential, as indicated by covalently-bound aminoacridines [22], and a fluorescamine-indicated conformational change of the chloroplast ATPase [23]. Of particular interest is the observation that the onset of the oxonol VI shift is approximately concomitant with the fast phase of the carotenoid-shift decay, the slow phase coinciding with the decay of the oxonol VI shift. This observation is at variance with an interpretation of the carotenoid-shift decay kinetics forwarded in [9]. There, it was proposed that the fast phase of the decay is due to rapid dissipation of the light-induced $\Delta\psi$ in damaged chloroplasts and the slow phase to the $\Delta\psi$ decrease in intact chloroplasts. From the data presented in fig.1–3, however, it appears that the light-induced decrease in oxonol VI A_{590} follows the production of $\Delta\psi$ and not its breakdown. An alternative explanation is that the carotenoid shift monitors an intramembrane, energy-conserving event prior to $\Delta\psi$ formation as suggested [18,24]. The conversion of this to a bulk $\Delta\psi$ may then be reflected by the rapid phase of the carotenoid-shift decay and by the onset of the oxonol VI shift. The slow phase of the carotenoid-shift decay and the decay of the oxonol VI shift may then monitor the dissipation of the $\Delta\psi$.

The magnitude of the flash-induced $\Delta\psi$, as measured from the size of the oxonol VI shift, and the calibration curve in fig.3, was 50 mV. This agrees well with values obtained using the carotenoid shift technique (e.g., 50 mV [9] and 35 mV [13]) and with some values reported from studies using micro-electrodes (e.g., 40 mV [21] and 50–75 mV [25]), although much lower values have also been obtained with the latter technique (e.g., 4–12 mV [26,27]).

It is clear that oxonol VI responds quantitatively to $\Delta\psi$ in chloroplasts, and that it can supplement existing techniques in providing valuable data on $\Delta\psi$ formation during the early stages of photosynthesis. Questions remain concerning the mechanism and kinetics of the probe response and these are presently under investigation in this laboratory.

Acknowledgements

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References

[1] Jagendorf, A. T. and Uribe, E. (1966) Proc. Natl. Acad. Sci. USA 50, 170–177.
[2] Schuldiner, S., Rottenberg, H. and Avron, M. (1973) Eur. J. Biochem. 39, 455–462.
[3] Uribe, E. and Li, B. C. Y. (1973) J. Bioenerget. 4, 35–44.
[4] Junge, W. (1977) Ann. Rev. Plant. Physiol. 28, 503–536.
[5] Mitchell, P. (1966) Biol. Rev. 41, 445–502.
[6] Mitchell, P. (1974) FEBS Lett. 43, 189–194.
[7] Rottenberg, H., Grunwald, T. and Avron, M. (1971) FEBS Lett. 13, 41–44.
[8] Rottenberg, H., Grunwald, T. and Avron, M. (1972) Eur. J. Biochem. 25, 54–63.
[9] Witt, H. T. (1971) Quart. Rev. Biophys. 4, 365–477.
[10] Harris, D. A. and Crofts, A. R. (1978) Biochim. Biophys. Acta 502, 87–102.
[11] Rüppel, H. and Witt, H. T. (1969) Methods Enzymol. 16, 316–380.
[12] Schmidt, S., Reich, R. and Witt, H. T. (1971) Naturwissenschaften 8, 414–423.
[13] Schapendonk, A. H. C. N. and Vredenberg, W. J. (1977) Biochim. Biophys. Acta 462, 613–621.
[14] Vredenberg, W. J. (1976) in: The Intact Chloroplast (Barber, J. ed) Topics in Photosynthesis, vol. 1, pp. 53–88, Elsevier/North-Holland Biomedical Press, Amsterdam, New York.
[15] Waggoner, A. (1976) J. Membr. Biol. 27, 317–334.
[16] Cohen, L. B., Salzberg, B. M., Davilla, H. V., Ross, W. N., Landowne, D., Waggoner, A. and Wang, C. H. (1974) J. Membr. Biol. 19, 1–25.
[17] Schuurmans, J. J., Casey, R. P. and Kraayenhof, R. (1978) unpublished observations.
[18] Chance, B. and Baltscheffsky, M. (1975) Biomembranes 7, 33–59.
[19] Bashford, C. L. and Thayer, W. S. (1977) J. Biol. Chem. 252, 8459–8463.
[20] Terada, H., Uda, M., Okitsu, T., Kametani, F. and Kubota, S. (1970) FEBS Lett. 78, 77–80.
[21] Bulychev, A. A., Andrianov, V. K., Kurella, G. A. and Litvin, F. F. (1976) Biochim. Biophys. Acta 430, 336–351.
[22] Kraayenhof, R. and Arents, J. C. (1977) in: Electrical Phenomena at the Biological Membrane Level (Roux, E. ed) pp. 493–504, Elsevier, Amsterdam, New York, Oxford.
[23] Kraayenhof, R. and Slater, E. C. (1974) in: Proc. 3rd Int. Congr. Photosynthesis (Avron, M. ed) pp. 985–996, Elsevier, Amsterdam, New York, Oxford.
[24] Sewe, K. U. and Reich, R. (1977) FEBS Lett. 80, 30–34.
[25] Bulychev, A. A., Andrianov, V. K., Kurella, G. A. and Litvin, F. F. (1972) Nature 236, 175–176.
[26] Vredenberg, W. J., Homann, P. H. and Tonk, W. I. M. (1973) Biochim. Biophys. Acta 314, 261–265.
[27] Vredenberg, W. J. and Tonk, W. J. M. (1974) FEBS Lett. 42, 236–240.