ESTIMATION OF THE LIGHT-INDUCED ELECTRICAL POTENTIAL AT THE FUNCTIONAL MEMBRANE OF PHOTOSYNTHESIS USING A VOLTAGE-DEPENDENT IONOPHORE*

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1. Introduction

A light-induced electrical field generation across the functional membrane of photosynthesis – so-called thylakoid membrane – has been demonstrated by electrochromism. This method is based on the shift of the absorption bands of the membrane bulk pigments in an electrical field [1–3]. The corresponding absorption changes have been proved to indicate a field by 3 different types of experiments: kinetic [1], spectroscopic [3,4] and electric [5]. The field-indicating absorption changes have the following properties: (1) The indication is 'instantaneous' (< 20 ns) [6]. (2) The absorption changes respond to potential differences adjusted perpendicular to the thylakoid membrane [5,7]. (3) They are linearly proportional to the indicated potential difference [8,9]. (4) The absorption changes were calibrated in volts [8]. These properties are useful for measuring the electrical events in membranes in general and for analysing whether the energy of the field can be used for phosphorylation (see review [10]).

For quantitative analysis a correct calibration of the absorption changes is necessary. This is of special importance in respect to the question whether the electrochemical potential of the protons – depending on the transmembrane voltage and pH gradient – can satisfy the energetic requirement for ATP synthesis [11]. In a first approach the number of translocated charges, Q, per flash across the thylakoid membrane of green plants were measured and assumptions were made on the capacitance C of the membrane. It resulted for a single turnover electron transfer the generation of a transmembrane voltage of $\Delta\phi_0 \approx 50$ mV [8].

In a second approach diffusion potentials were set across the inner membrane of bacteria by salt jumps and it was assumed that the Nernst equation is valid [12]. It resulted in bacteria for a single turnover electron transfer approx. 100 mV [13]. The last method is not applicable to the thylakoid membranes of green plants because, in this case, changes in light scattering prevent electrochromic measurements [14, 15]. It is therefore, desirable to have a second method for voltage calibration at thylakoids. The use of alamethicin as a voltage-dependent ionophore is a possibility. Our communication deals with relevant experiments.

2. Materials and methods

Chloroplasts were prepared by a method described elsewhere [16]. For the measurements we used freshly

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prepared spinach chloroplasts. The measuring suspensions contained 10^{-5} M chlorophyll, 10^{-4} M benzylviologen, 10^{-2} M sucrose, 10^{-2} M tricine at pH 8 and chloride salts of different concentrations (10^{-2} – 10^{-3} M). Alamethicin was kindly provided by Dr Whitfield, Upjohn Co., Kalamazoo, Michigan, and Professor Dr Kleinkauf from our institute. It was used in concentrations varying from 10^{-7} g/ml to $2 \cdot 10^{-5}$ g/ml. All reagents were suspended in the solution before adding the chloroplasts. The field-indicating absorption changes were measured at 515 nm with a repetitive flashlight photometer described elsewhere [17]. The measuring light intensity was $I \approx 10\ \mu W/cm^2$. The signal-to-noise ratios were improved by averaging the signals. Normally 50 flashes were sampled. The frequency of flash repetition was below 0.5 Hz in order to avoid the generation of a light-induced base potential. Wavelength of excitation was $\lambda > 610$ nm (Schott RG 610). Saturating single turnover flashes (half lifetime 20 μs) and saturating double flashes (spaced by 2 ms) were used. All measurements were performed at room temperature (~ 23°C).

3. Results and discussion

In artificial lipid membranes alamethicin is known to act as a voltage dependent pore former [18–24, 31,32]. Alamethicin molecules, adsorbed at the membrane–solution interface, probably form a complex with a cation from the solution, whereby part of the primary hydration shell of the ion is replaced by amide carbonyls of the peptide. When a positive voltage is applied to the membrane at that side where the alamethicin is adsorbed, the positively charged end of the complexed molecule (gating charge) is turned into the hydrophobic interior of the membrane while the glutamic C-terminal end remains attached to the initial surface. In this state after cation dissociation the molecules possess a tendency to aggregate, forming dimers, trimers, tetramers, etc.

The interior of aggregates of more than two alamethicin molecules becomes a hydrophylic pore for ions whereas the hydrophobic residues of the aminoacids are pointing outwards into the lipids [20,21]. In this state the conductance of the membrane is drastically increased. The pores are relatively large in diameter (10–20 Å) and therefore do not show strong selectivity for different types of ions [18,19]. The mean voltage at which, under given conditions, the formation of one pore is certain to occur has been labelled 'characteristic voltage', $\Delta\phi_c$, [19]. It was shown that $\Delta\phi_c$ is an appropriate variable to characterize the properties induced by alamethicin in lipid membranes. The characteristic voltage, $\Delta\phi_c$, depends on the concentration of alamethicin as well as of the ions.

Under appropriate current-clamp conditions the generation of oscillation phenomena induced by the voltage-dependent formation and disappearance of alamethicin pores has been shown [24]. In addition, it was observed that alamethicin slightly increases the basal conductivity also without an applied voltage [23,29].

We asked whether alamethicin is also effective on thylakoid membranes as it has been reported for the plasma membrane of *A. laidlawii* [25].

The principle effect of alamethicin on thylakoid membranes is illustrated in fig.1. In fig.1A the signal of the field-indicating relative absorption change at 515 nm, $\Delta A/\Delta A_0$, induced by a saturating double-flash without alamethicin is plotted ($\Delta A/\Delta A_0$ is the absorption change ΔA in relation to the total change ΔA_0 in a saturating single turnover flash). It decays with $\tau \approx 250$ ms. The addition of alamethicin changes the signal into a biphasic decay with a fast phase of $\tau \approx 10$ ms and a slow one of $\tau \approx 150$ ms (see fig.1B). The effect can be explained in two ways:

(1) It is assumed that alamethicin is homogeneously distributed on the thylakoid membrane. The saturating double-flash induces an electrical potential difference across the membrane which is above the characteristic voltage $\Delta\phi_c$. It induces the formation of alamethicin pores in the membrane. As a consequence of the attributed increase of conductance the field decreases rapidly ($\tau \approx 10$ ms). When thereby the characteristic voltage $\Delta\phi_c$ is passed, the pores mainly disappear with a slow field decay of $\tau \approx 150$ ms remaining.

(2) It is assumed that alamethicin acts practically permanently during the whole decay time of the signal as an ionophore, i.e. independently of the voltage. If under these conditions alamethicin is inhomogeneously distributed among the thylakoids, e.g. part of the thylakoids is occupied by more alamethicin than the others, one would observe a fast and a slow signal

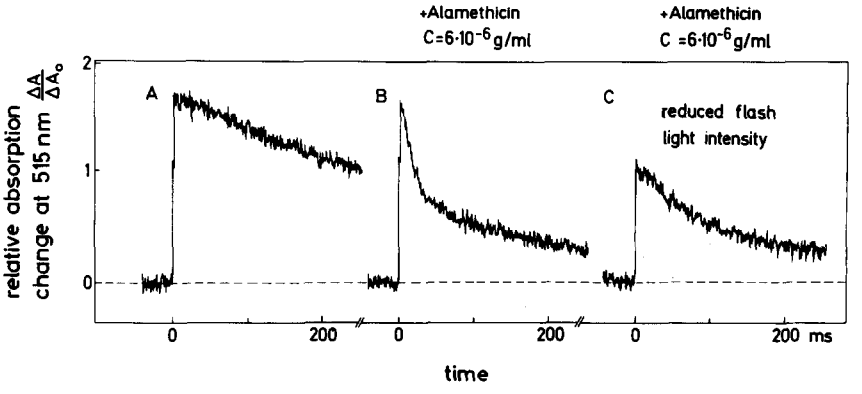

Fig.1. Field-indicating absorption changes at 515 nm (relative in a linear plot) as a function of time. (A) without and (B) with $6 \cdot 10^{-6}$ g/ml alamethicin, induced by a saturating double flash, (C) with $6 \cdot 10^{-6}$ g/ml alamethicin, induced by a saturating single turnover flash. Subject: Spinach chloroplasts.

decay. The overlap of both types of signals would result in an apparent biphasic decay. Such effects were demonstrated for the ionophores valinomycin and nonactin at thylakoid membranes [42].

In order to discriminate between these possibilities we have decreased the total voltage homogeneously for all thylakoids below the level of the break. Under these conditions we expect in case (1) a reduced signal with the slow decay phase only and in case (2) a reduced signal but with the same biphasic shape as under conditions of high flash intensity. The result is shown in fig.1C. The suspension is illuminated with a saturating single turnover flash instead of a saturating double flash as shown in fig.1B. Under these conditions in fig.1C practically only the slow phase of fig.1B is observed. Obviously case (1) is realized, i.e. the electrical potential difference does not exceed the characteristic voltage $\Delta\phi_c$. Therefore, almost no pores are formed by alamethicin and the field decay is slow. A homogeneous decrease of the voltage below the level of the break at a saturating double flash is also possible by blocking one of the photoactive centers. We blocked photosystem II by adding 10^{-6} M 3-(3,4-dichlorphenyl)-1,1 dimethylurea. Then, in the presence of 10^{-4} M dichlorphenol–indophenol and 10^{-2} M ascorbate only photosystem I acts as an electron translocator across the membrane and only one half of the transmembrane voltage is induced [8]. Such measurements gave the same results in the presence of alamethicin as those in fig.1B and 1C.

Because of these results we assume that alamethicin acts in thylakoid membranes similarly as in artificial bilayers, i.e. the observed level of the break between the fast and the slow phase of the field-indicating absorption change ΔA_c corresponds to the characteristic voltage $\Delta\phi_c$. This interpretation is supported by the result in fig.2 which shows the different action of

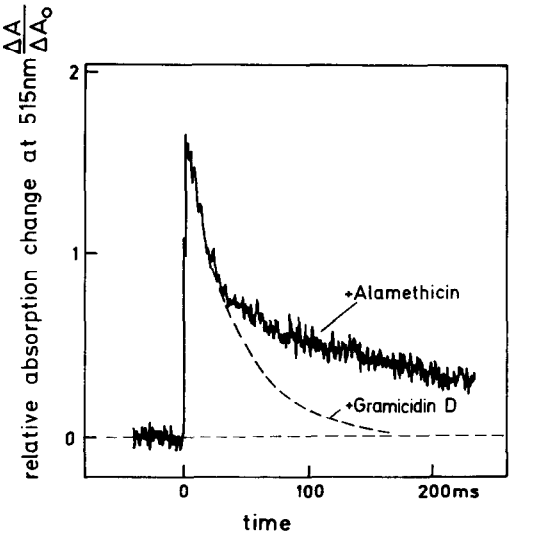

Fig.2. Field-indicating absorption change at 515 nm (relative in a linear plot) as a function of time (same curve as in fig.1B with $6 \cdot 10^{-6}$ g/ml alamethicin). The dashed line indicates the time course in the presence of gramicidin D ($\approx 6 \cdot 10^{-11}$ M) instead of alamethicin. Subject: Spinach chloroplasts.

alamethicin and gramicidin D on chloroplast membranes. Gramicidin D is known to act as an ionophore independent of the transmembrane voltage. In contrast to alamethicin the action of gramicidin D causes, under the condition of a homogeneous distribution, a monophasic exponential decay. After the flash the thylakoid membrane is negatively charged on the outside and positively inside [1,5]. Because of the observed action of alamethicin on thylakoids (see fig.1) it follows that alamethicin must be present also in the inner thylakoid space. This may be plausible considering the fact that the presence of unsaturated hydrocarbons in lipids of artificial membranes – which are also present in thylakoids [26] – increases the ease of alamethicin diffusion across membranes [18,19]. But we cannot rule out the possibility that alamethicin pore formation may occur also from the negative potential side of the membrane [31]. It should be noted that in solutions which contain additional $CaCl_2$ ($10^{-3} - 10^{-2}$ M) (a) the break between the two phases in fig.1B is more pronounced and (b) a certain level of the break can be realized by a lower concentration of alamethicin than in the absence of $CaCl_2$. Both effects might be due to the aggregation effect induced by $CaCl_2$ in lipid membranes [27,28]. This aggregation leads probably on the one hand to 'solid' membrane areas and on the other hand to 'fluid' regions. This 'fluid' region may allow (a) a more homogeneous distribution of alamethicin among the different thylakoids and (b) a higher concentration of alamethicin in the inner space of the thylakoids.

Additionally it should be noted that the possible influence of a light-induced transmembrane pH gradient on $\Delta\phi_c$ was ruled out by adding in a series of experiments ammonium chloride in a concentration of 10^{-3} M. No significant differences in respect to $\Delta\phi_c$ were observed in relation to experiments without this uncoupler.

The characteristic voltage $\Delta\phi_c$ is related to the alamethicin concentration C as follows [19,21]:

$$\Delta\phi_{c_1} - \Delta\phi_{c_2} = \frac{1}{\alpha}\,\frac{RT\ 2.3}{F}\log\frac{C_2}{C_1} \qquad (1)$$

α is a constant and varies only with the structure and material of the membranes. In artificial lipid bilayers α ranges from 0.72 to 0.93 [19,21].

Eqn. (1) provides a possibility of calibrating the field-indicating absorption changes if we assume that the concentration of alamethicin in the inner thylakoid space is proportional to the concentration in the suspension.

Fig.3 shows the result at two concentrations of alamethicin which differ by a factor 10. Because the absorption changes ΔA are related linearly to the

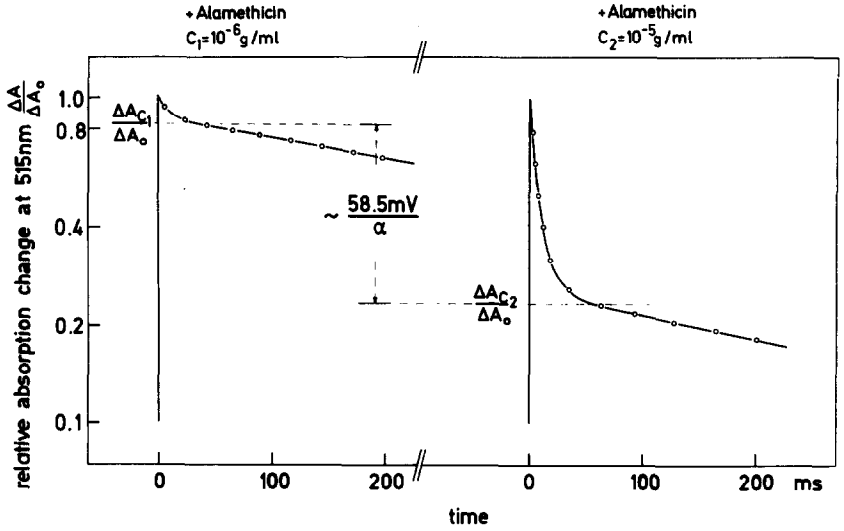

Fig.3. Field-indicating absorption changes at 515 nm (relative in a log-plot) as a function of time at two different concentrations of alamethicin and induced by a saturating single turnover flash. Subject: Spinach chloroplasts.

voltage [8,9], the difference ($\Delta A_{c_1} - \Delta A_{c_2}$) between the level of the breaks which is $0.6 \cdot \Delta A_0$, must correspond to $(1/\alpha) \cdot (R \cdot T \cdot 2.3/F) \cdot \log 10 = (1/\alpha) \cdot 58.5$ mV, i.e. with $\alpha = 0.72$–0.93 the value of ΔA_0 should indicate a voltage of 105–135 mV. In fig.4 the difference of the characteristic absorption changes, $\Delta A_{c_1} - \Delta A_{c_2}$, in relation to the total change in a saturating single turnover flash, ΔA_0, is plotted as a function of the ratio of different alamethicin concentrations, $\log c_2/c_1$ to prove whether eqn. (1) can be applied to thylakoids in general. The different points refer to different salts. The result is in fair agreement with a logarithmic dependence on the concentration. Because the absorption changes are related linearly to $\Delta\phi$, i.e. $\Delta\phi = b \cdot \Delta A$, it is $\Delta\phi_c = b \cdot \Delta A_c$. With eqn. (1) and $\Delta A/\Delta A_0$ (absorption change ΔA in relation to ΔA_0 in a saturating single turnover flash) it follows at 23°C:

$$\Delta\phi_{c_1} - \Delta\phi_{c_2} = b \left(\frac{\Delta A_{c_1}}{\Delta A_0} - \frac{\Delta A_{c_2}}{\Delta A_0}\right) \Delta A_0 = \frac{1}{\alpha} \cdot 58.5 \text{ mV} \cdot \log \frac{c_2}{c_1} \qquad (2)$$

The value of b and the slope m of the curve in fig.4 are related as

$$b = \frac{58.5 \text{ mV}}{\alpha \cdot m \cdot \Delta A_0}.$$

In fig.4 it is m = 0.6. With this value for m we get:

$$b \approx \frac{100 \text{ mV}}{\Delta A_0 \cdot \alpha} \qquad (3)$$

With b we calculate the maximal voltage $\Delta\phi_0$ in a saturating single turnover flash which induces an absorption change ΔA_0. For $\alpha = 0.72$–0.93 it is,

$$\Delta\phi_0 = b \cdot \Delta A_0 \approx 105\text{–}135 \text{ mV}. \qquad (4)$$

The value of $\Delta\phi_0$, obtained by the method described above, is more than two times larger than that based on the capacitor method cited in the Introduction [8]. It is therefore necessary to discuss the uncertainties of that estimation. The potential $\Delta\phi_0$ was calculated according to $\Delta\phi_0 = Q_0/C_m \cdot Q_0$ is the number of charges translocated across the membrane per electron transfer chain and C_m is the capacitance of the membrane which is given by $C_m = (\epsilon \cdot \epsilon_0 \cdot S)/l$. ϵ is the dielectric constant of the membrane, ϵ_0 the dielectric

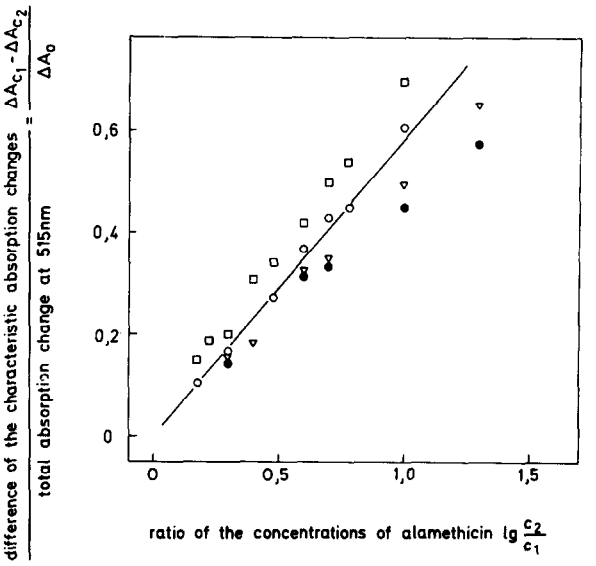

Fig.4. Difference of the characteristic field indicating absorption changes, $\Delta A_{c_1} - \Delta A_{c_2}$ in relation to the total absorption change, ΔA_0, (linear plot), as a function of the ratios of different alamethicin concentrations, (log plot). Subject: Spinach chloroplasts in different solutions of salts. (□–□–□) $4 \cdot 10^{-3}$ M $CaCl_2$ (○–○–○) and (▽–▽–▽) 10^{-2} M KCl (●–●–●) 10^{-2} M NaCl.

constant of vacuum, S the surface area per electron transport chain and l the thickness of the membrane lipid layer. S was obtained from the amount nth of chlorophyll per thylakoid, the amount n_e of chlorophyll per electron transport chain and the surface are A per thylakoid, i.e. $S = A \cdot n_e/nth$. If we assume that Q_0, A and ϵ are rather well known ($Q_0 \approx 2e$ (8), $A \approx 4 \cdot 10^7$ Å^2 (38) and $\epsilon \approx 2$ for a lipid layer [43]) the value for $\Delta\phi_0$ ranges according to the figures listed in table 1 from $\Delta\phi_0 \approx 30$ mV to $\Delta\phi_0 \approx$ 90 mV. We excluded the value l = 21 Å which corre-

Table 1
Values for the thickness of the thylakoid membrane, l, the number of chlorophylls per electron transport chain, n_e, and the number of chlorophylls per thylakoid, nth. (references in brackets).

l (Å)	21 [41],	35 [30],	40 [34,35]
n_e	400–600 [36],	570 [37]	
nth	$1.1 \cdot 10^5$ [38],	$1.6 \cdot 10^5$ [39]	$1.8 \cdot 10^5$ [33]

sponds to a lipid monolayer. The value $\Delta\phi_0 \approx 50$ mV reported in [8] is an averaged value obtained with figures of 1, n_e and nth which were available to us at that time.

According to this work we get $\Delta\phi_0 \approx 105-135$ mV (with $\alpha = 0.72-0.93$). Comparing this new result with the older one (30–90 mV), we may conclude that the higher value is the more probably one , i.e. $\Delta\phi_0 \approx 100$ mV. Nevertheless, it seems desirable to have results from an independent third method. However, at the moment no results from other methods are available. Measurements with microcapillary glass electrodes inserted in a single grana stack indicate a single turnover potential of about 10 mV [44,45] *. However, these results cannot be considered because the hole which is produced by the insertion (radius ~ 0.5 μm, i.e. a hole of the size of about a whole thylakoid) must falsify the true potential considerably. This is indicated already by the decay time which is 10 times faster ($\sim$ 10 ms) [45] than without electrodes ($\sim$ 100 ms) [1]. Light-induced potentials calculated from measurements of delayed light emission [46] were not carried out at single turnover conditions and can therefore also not be used for a comparison.

Besides the use of alamethicin for calibration it is shown by the above results that it is possible to incorporate into an in vivo membrane an artificial switch which responds to a light-induced electrical field.

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*Note added in proof

Recently a paper has been published with a refined value of $\sim$ 40 mV [40].

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