Biochimica et Biophysica Acta, 681 (1982) 202–211
Elsevier Biomedical Press

BBA 41111

SALT DEPENDENCE OF THE ELECTRICAL POTENTIAL AT THE PHOTOSYNTHETIC MEMBRANE IN STEADY-STATE LIGHT AND ITS STRUCTURAL CONSEQUENCE *

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(Received December 21st, 1981)

Key words: Salt effect; Photosynthesis; Electrochromic absorption change; Transmembrane potential difference; Membrane structure; (Spinach chloroplast, Thylakoid)

(1) The change in potential difference across the functional membrane of photosynthesis has been measured as a function of KCl concentration of the suspending medium under saturating, continuous illumination. The measurements have been carried out through the potential-indicating electrochromic absorption changes. Between 1 and 100 mM KCl, a decrease from 80 to about 0 mV was found. The salt dependence can be described by a Donnan model which is based on buffer groups located within the inner space of the system. A Gouy-Chapman model based on buffer groups located at the plane of the inner membrane surface does not fit the data of the measurements. The strong salt dependence may explain apparent discrepancies between the values of the light-induced potential changes reported in the literature. (2) Measurements of the membrane conductance of the ionophore, nonactin-K^+, as a function of the internal proton concentration have been carried out. Also, in this case, the results can be explained by a Donnan model. The consequence of both results, i.e., distribution of the buffer groups of thylakoids within the inner space, is possibly realized by membrane-bound proteins which project up to about 20 nm into the inner thylakoid space, as well as through unbound proteins (see Fig. 7). Inter alia, the carboxyl groups of aspartic and glutamic acids of these proteins may be the effective buffer groups. Plastocyanin may also contribute to this effect in a minor way.

Introduction

A vectorial electron transfer from the inside to the outside of the thylakoid membrane has been detected through electrochromic absorption changes [1]. This charge separation generates a corresponding electrical potential difference across the membrane. Refined analysis of the electrochromic absorption change within the last 15 years led to (a) evaluation of the electric properties of the membrane, (b) information about the mechanism of phosphorylation, and (c) insights into the molecular organization of the membrane (for details see Ref. 2). In this paper, analysis of the steady-state electric potential difference through the electrochromic method is used to obtain further topographical information, especially on the structure of the inner thylakoid space. The primary electron shift sets off a transmembrane voltage of approx. 50 mV [2]. Proteolytic reactions of the primary separated charges result in inward H^+ translocation. With saturating, continuous light, uptake of about 0.3 H^+/Chl is achieved which induces a change in the steady-state acidification in the inner thylakoid space from pH in 8 to 5 [3].

* This work has already been published in abstract form [7].

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Abbreviations: Chl, chlorophyll; Tricine, *N*-tris(hydroxymethyl)methylglycine; FCCP, carbonyl cyanide *p*-trifluoromethoxyphenylhydrazone.

Most of the inwardly pumped H^+ (approx. 99%) is bound by negative buffer groups, A^-. Location of the buffer groups, either on the inner membrane surface (Gouy-Chapman type) or within the inner thylakoid space (Donnan type), is a possible alternative. Buffer groups on the surface but projecting into the inner space may be a more realistic model (see Discussion). Due to H^+ binding, the charge density of the inner thylakoid space is shifted toward more positive values. This results in a change, $\Delta\psi$, in the transmembrane electric potential difference as schematically depicted in Fig. 1. This steady-state potential was first interpreted by Schröder et al. [5] as being based on a Donnan type but later as based on an extended Gouy-Chapman type [6] (see Discussion).

In principle, the active H^+ fluxes have to be considered in the calculation of the steady-state potential difference. But, since in chloroplasts the concentration of H^+ is at least 100-times smaller than the bulk concentrations of the other ions, H^+ flux can be neglected. Hence, the steady-state potential can be calculated reasonably well regarding an equilibrium state. Therefore, the more convenient Donnan and Gouy-Chapman theories have been used in this study.

A distinction between the two models of binding corresponding to a Donnan and a Gouy-Chapman potential is possible by their different dependences on the ion concentration of the aqueous phase. Such experiments have been carried out by us through measurements of the extent of electrochromic absorption changes as a function of KCl concentration. Experiments of this kind were started in our laboratory by Zickler (1977, unpublished results). The extension of our measurements was published in a first report in 1979 [7]: The change in the light-induced transmembrane potential difference, $\Delta\psi$, is from 0 to 80 mV in the steady state on altering of the KCl concentration from 100 to 1 mM. Additionally, the membrane conductance of the ionophore, nonactin-K^+, has been measured through the kinetics of the electrochromic absorption change as a function of pH_{in}, thereby titrating the membrane potential as a function of pH_{in}. In both cases a Donnan-like potential fits the measured dependence. As a consequence of these results one has to conclude that the buffer substances are distributed over the inner space. These substances are possibly unbound proteins and membrane-bound proteins which are projecting, with their buffer groups, into the whole inner thylakoid space.

Materials and Methods

Spinach chloroplasts were prepared according to the method of Winget et al. [8] except that 10 mM ascorbate was present during the grinding of leaves. These chloroplasts were washed twice in distilled water in order to remove most of the ions of the preparation solution and were used immediately. The stock chloroplast suspension was diluted in an aqueous solution of 0.5 mM (1 mM in the conductivity measurements) Tricine-NaOH buffer (pH 8), 100 μM benzyl viologen, and KCl at different concentrations (see figure legends) to obtain a chlorophyll concentration of 10 μM. The reaction volume was 10 ml. For H^+ measurements at pH 8, 25 μM cresol red was added. When nonactin or FCCP, dissolved in methanol, was added care was taken to keep the final concentration of methanol below 0.5%. This concentration does not affect photosynthesis. The measurements were carried out at room temperature (18–21°C). The reaction cuvette was placed into a repetitive flash photometer as described earlier [9]. The optical path length of the cuvette was 2 cm. The bandwidth of the measuring beam was $\Delta\lambda = 5$ nm, the intensity of the detecting light was less than 100 $erg \cdot cm^{-2} \cdot s^{-1}$, and the electric bandwidth of the detection system was 30 kHz.

Photosynthesis was initiated either with repetitive single turnover flashes ($\tau_{1/2} \approx 20$ μs) or with continuous light of 6 s duration. The actinic light passed through red filters (Schott RG 610 2 mm), the measuring light through Schott interference filters (DAL 520, electrochromism or DAL 571, H^+ measurements). To improve the signal-to-noise ratio, between 1 and 64 signals were averaged. The repetition rates of the single turnover flashes were 1 Hz (electrochromism) and 0.04 Hz (H^+ measurements).

Steady-state potential measurements

Artifacts due to exciting light scattered by the turbid chloroplasts suspension were avoided by a

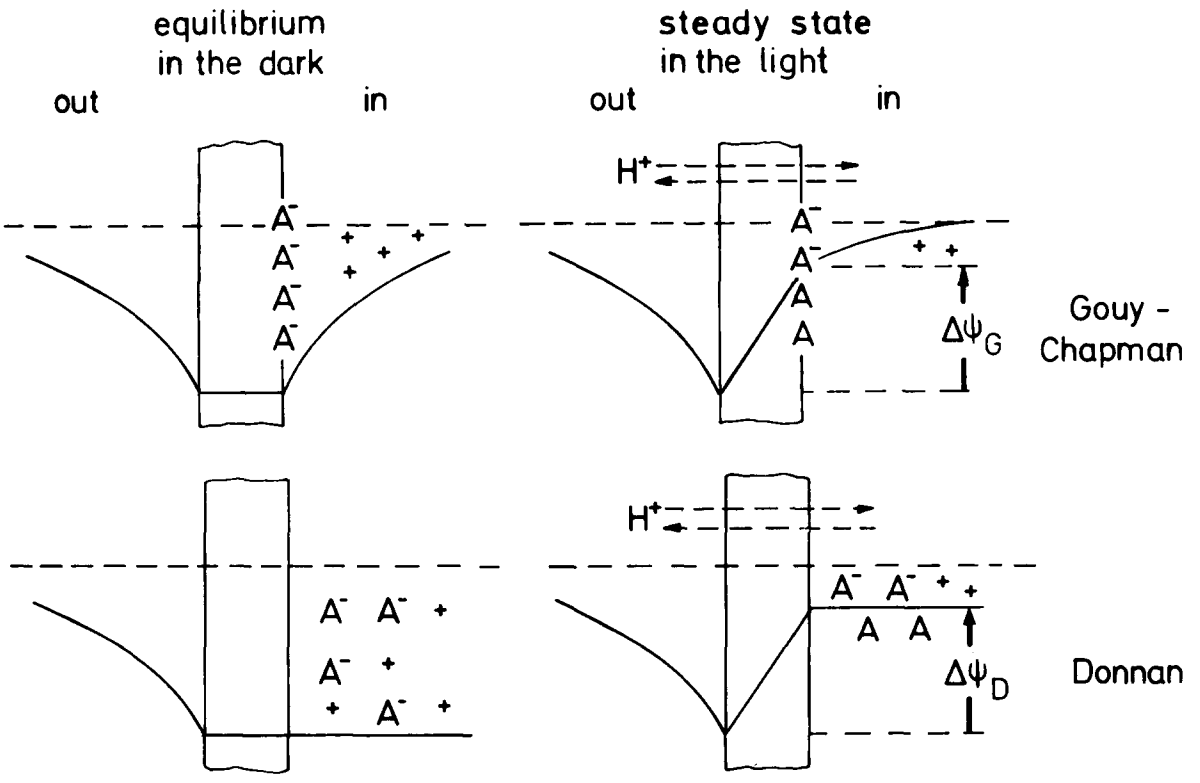

Fig. 1. Scheme of the light-induced change of the transmembrane potential difference in the case of an inner Gouy-Chapman and an inner Donnan potential. The dark states are shown on the left and the illuminated ones on the right of the figure. The buffer groups of the inner phase are indicated by A. For the outer phase a Gouy-Chapman potential is assumed.

combination of guard filters (Schott DAL 520 and BG 38/4) placed in front of the cathode of the photomultiplier (EMI 9558 BQ). A more serious problem is caused by artifacts resulting from transient changes of the light-scattering properties of the sample on excitation, especially in the range of seconds to minutes [10,11]. This problem was solved by measuring with a large aperture. Thus, nearly all light from the cuvette (scattered and transmitted) was focussed on the cathode of the photomultiplier. The success of this procedure was checked by measuring the absorption change at 494 nm where the difference spectrum of the field-indicating absorption change has a zero point. At this wavelength no significant effect due to changes in scattering properties of the sample could be monitored. The absorption change at 520 nm measured under these conditions was, therefore, directly correlated with the electrochromism. It should be noted here that this experimental setup for elimination of the scattering artifacts is only successful when the reaction medium contains very small amounts of magnesium (the large transient scattering measured by Thorne et al. [10] is mainly attributable to the magnesium in the reaction medium). Scattering has been completely avoided by using single turnover flashes in combination with conductance measurements (see below). Since the results of such experiments are the same as those carried out with continuous light, this supports the conclusion that scattering effects under continuous light have been excluded. The field-indicating absorption changes at 520 nm under steady-state illumination were calibrated using the corresponding absorption changes for single turnover excitation, which reflect a change of 50 mV in the transmembrane voltage (for a review of the different calibration procedures and various values of the voltage change in single turnover flashes see Ref. 2). The shift in the transmembrane potential difference, $\Delta\psi$, and the absorption change, ΔA, are related as follows:

$$\Delta\psi(t) = 50 \cdot \Delta A(t)/{}^{1}\Delta A \text{ mV} \quad (1)$$

where $\Delta A(t)$ denotes the change in absorption at time t after the onset of illumination and ${}^{1}\Delta A$ the maximum change in absorption in single turnover flashes at $t = 0$.

Conductance measurements in single turnover flashes

The steady-state membrane potential of the thylakoids can be titrated by measurements of the membrane conductance. The use of neutral carriers of cations and anions in the study of the membrane conductance of phospholipid membranes is well established (for a review see Ref. 37). The applicability of valinomycin and nonactin to chloroplasts has been thoroughly studied by Junge and Schmid [38,39]. We used nonactin as carrier of K^+ and measured the membrane conductance via the electrochromic absorption changes in short flashes. From Eqn. 1 it follows that the slope of the changes represents the current, $I(t)$:

$$I(t) = 3.2 \cdot 10^{-8} \cdot \frac{d}{dt}\left(\Delta A(t)/{}^{1}\Delta A\right) \text{ A} \cdot \text{cm}^{-2} \quad (2)$$

The membrane conductance is then defined as:

$$G(t) = \frac{I(t)}{\Delta\psi(t)} = 6.4 \cdot 10^{-7} \cdot \frac{1}{\Delta A(t)} \cdot \frac{d(\Delta A(t))}{dt} \ \Omega^{-1} \cdot \text{cm}^{-2} \quad (3)$$

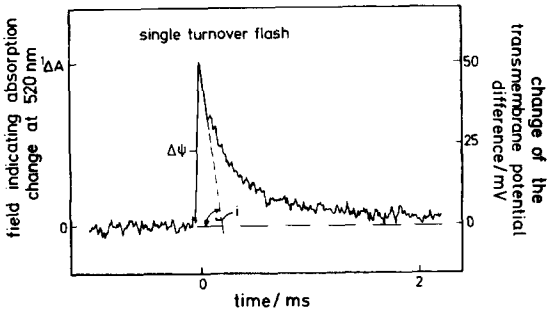

Fig. 2. Time course of the field-indicating absorption change, ΔA, at 520 nm after excitation by a single turnover flash. The rapid rise of absorption (less than 1 ns) indicates the induction of an electric potential difference across the membrane by charge separation. The relaxation of the absorption change reflects, the discharging of the membrane. Evaluation of the voltage, $\Delta\psi$ (corresponding to the extent of $\Delta A(t)$) and current density, I (corresponding to the slope of $\Delta A(t)$) has been carried out according to Eqns. 1 and 2.

The determination of I(t) and $\Delta\psi(t)$ and thus $G(t)$ at $t = 0$ is illustrated in Fig. 2.

$G(t)$ is the sum of the membrane conductance for the ionophore complex and the intrinsic conductance of the thylakoid membrane. An experiment without the addition of nonactin has to be performed in order to separate the membrane conductance for the ionophore complex

$$G_{\text{nonactin-K}^+} = G \text{ (with nonactin)} - G \text{ (without nonactin)} \quad (4)$$

$G_{\text{nonactin-K}^+}$ has been shown to be proportional to the K^+ concentration at the membrane surface [12]. In flashing light experiments, K^+ is transported from a position at the inner membrane surface across the membrane into the external aqueous phase. Therefore, the K^+ concentration at the inner membrane surface, c_s, has to be considered. A variation from c_{s1} to c_{s2} is correlated with a shift of the potential at the inner membrane surface from ψ_{s1} to ψ_{s2}, and thus with an alteration of the membrane conductance from G_1 to G_2:

$$\frac{c_{s1}^{+}}{c_{s2}^{+}} = \frac{G_1}{G_2} = \exp\left(\frac{F}{RT}\Delta\psi_s\right) \quad (5)$$

where $\Delta\psi_s$ denotes the change in the steady-state potential at the inner membrane surface (R, F and T have their usual meanings). An alteration of ψ_s and thereby of c_s at the inner surface, can be achieved by varying the H^+ concentration in the inner space, because the charged buffer groups of the thylakoid are controlled by proteolytic reactions (see Fig. 1). Measurements of the conductance were performed when the H^+ concentration of the inner thylakoid space was altered from $pH_{in} \approx 8$ up to $pH_{in} \approx 5$. This was achieved by preillumination of the chloroplast suspension with light of various intensities. The internal pH has been measured through the fluoresence quenching of 9-aminoacridine as described in Ref. 13. The H^+ concentration of the external phase was kept constant at pH_{out} 8 and also the outer KCl concentration. To determine the conductance, single turnover flashes were fired 100 ms after switching off the preillumination. This time lag is long enough to allow a relaxation of the reaction centers and short enough to keep the internal H^+ concentration constant.

The conductance, $G(t)$, with and without non-

actin was always determined according to Eqn. 3 at $t = 0$ immediately after firing the flash, i.e., $\Delta A(t = 0) = \Delta A^1$ (see Eqn. 1). This was done for two reasons: (1) The voltage built up across each thylakoid membrane is the same at $t = 0$. Therefore, permeability inhomogeneities within different thylakoids do not have to be taken into account. (2) The measurement of conductance has to be performed as soon as possible after switching off the preillumination in order to ensure that the conductance is determined at that H^+ concentration, pH_{in}, which is created by the preillumination.

H^+ uptake measurements

The pH-indicating absorption change of the indicator dye, cresol red, was calibrated using the absorption change on injection of a known amount of HCl (for details of H^+ measurements with the dye, cresol red, see Ref. 14). As cresol red does not penetrate the membrane, H^+ uptake from the external phase is measured by this method. Background absorption changes at 571 nm, which do not indicate pH changes of the external phase, were eliminated by subtracting the signals obtained in the absence from those obtained in the presence of cresol red.

Results

Salt dependence of the light-induced steady-state transmembrane potential difference

Fig. 3 shows a representative time course of the field-indicating absorption change under saturating continuous light excitation. Due to the primary charge separation, a fast rise of the electrochromic absorption change is monitored at the onset of illumination. The transmembrane voltage change, built up by the charge separation, triggers ion fluxes which tend to compensate this voltage. Thus, a decay in the electrochromic absorption change takes place until a steady-state level is reached. When the actinic light is switched off, the active H^+ influx stops immediately so that the passive H^+ efflux is no longer compensated. This effect causes a potential decay toward the initial level. The maximum amplitude, $^1\Delta\psi$, of the transmembrane potential difference built up during single turnover flash excitation was taken as a standard measure. The value of $^1\Delta\psi$ at various bulk concentrations, c_0, of KCl under excitation with single turnover flash light is shown in Fig. 4 (top). Also, in Fig. 4 (bottom), the external H^+ uptake under the same experimental conditions is depicted. The primary charge separation as well as the coupled H^+ uptake is independent of the salt content.

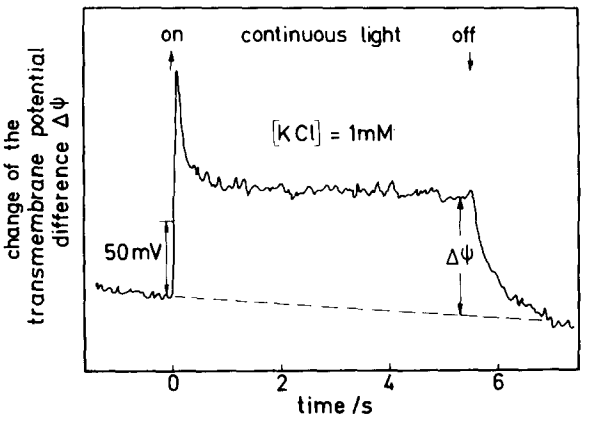

Fig. 3. Transmembrane potential difference as a funcion of time at 1 mM KCl induced by saturating continuous light.

The calibrated steady-state amplitudes of the electrochromic absorption changes under continu-

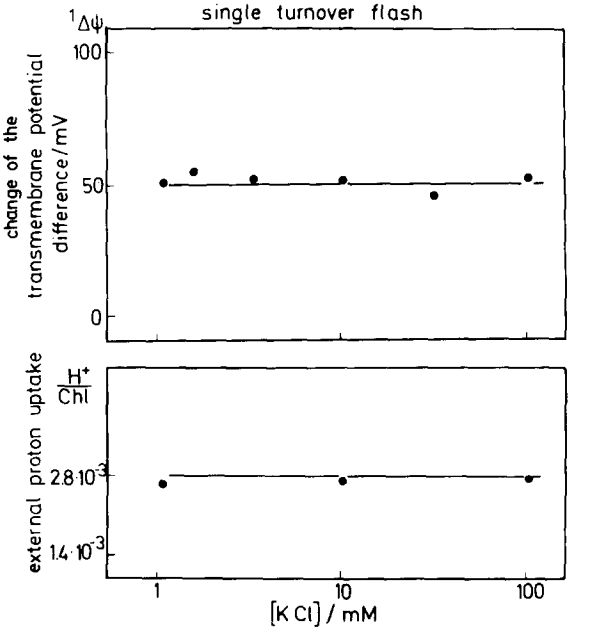

Fig. 4. (Top) Transmembrane potential difference induced by a single turnover flash as a function of the KCl concentration in the outer bulk phase. (Bottom) External H^+ uptake under the same conditions.

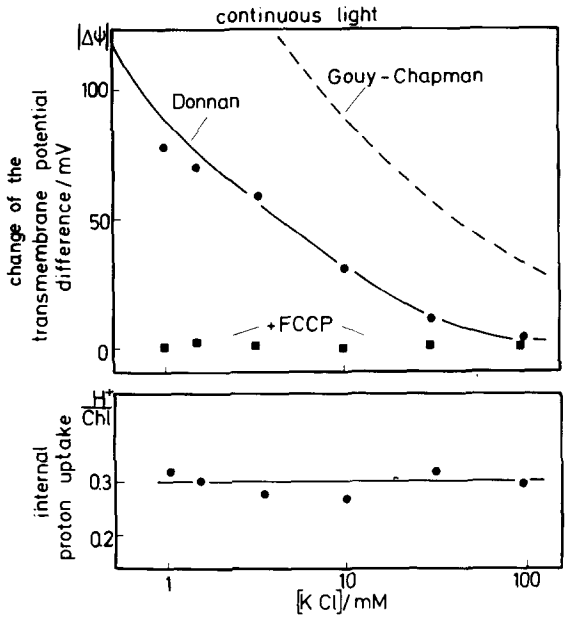

Fig. 5. (Top) Change of transmembrane potential difference in the presence (■) and absence (●) of an uncoupler (1 μM FCCP) as a function of the outer bulk concentration of KCl. The theoretical curves are calculated according to the Gouy-Chapman (- - - - -) and Donnan (———) theories, based on data discussed in the text. (Bottom) Steady-state H^+ uptake as a function of the bulk concentration of KCl. Excitation by saturating continuous light.

ous light excitation are plotted in Fig. 5 (top) as a function of the concentration of KCl. The circles indicate measurements without an uncoupler and the squares measurements in the presence of the uncoupler FCCP. In the absence of FCCP, a monotonic decrease in the steady-state potential difference from 80 to about 0 mV was monitored by increasing the salt concentration from 1 to 100 mM. With FCCP, the voltage is zero at all salt concentrations tested. Because FCCP makes the membrane permeable to H^+, this finding implies that the steady-state potential difference is caused by the action of H^+ within the thylakoid space. Knowledge of the H^+ uptake is essential for the theoretical description of the salt dependence of the steady-state potential. Therefore, in Fig. 5 (bottom), the light-induced steady-state H^+ uptake of the inner thylakoid space, which is supposed to determine the alteration of the internal charge density, has been measured as function of the KCl concentration. Independent of the salt concentration, a nearly constant H^+ uptake of 0.3 H^+/Chl was found in the cited range of concentrations. Due to this H^+ uptake, pH_{in} is shifted by three units from pH 8 to 5. The theoretical curves in Fig. 5 (top) are drawn according to the Gouy-Chapman theory [15] (dashed line) and the Donnan (solid line) theory [16]. The measured salt dependence of the steady-state voltage change is fairly well fitted by the theoretical Donnan trace. The traces are calculated with data (see below) for the surface area per chlorophyll molecule, S/Chl, the internal volume per mol chlorophyll, V_{in}/Chl, the density of the buffer groups, A^-, at the surface in the dark, σ_d, or the concentration of A^- in the inner space in the dark, A_d^-. In the light it is $\sigma_l = \sigma_d - \frac{\Delta H^+/\text{Chl}}{S/\text{Chl}}$ and $A_l^- = A_d^- - \frac{\Delta H^+/\text{Chl}}{V_{in}/\text{Chl}}$, respectively, ΔH^+/Chl is the H^+ uptake (0.3 H^+/Chl) in the light (Fig. 5) which is practically completely bound by the internal buffers (see Introduction). The change in the transmembrane potential difference from dark to light, $\Delta\psi_G$, is then according to the Gouy-Chapman model:

$$|\Delta\psi_G| = \frac{2RT}{F} \ln \frac{\sigma_l + \sqrt{\sigma_l^2 + 8RT\epsilon\epsilon_0 c_0}}{\sigma_d + \sqrt{\sigma_d^2 + 8RT\epsilon\epsilon_0 c_0}} \qquad (6)$$

where ϵ is the dielectric constant of water ($\epsilon = 80.1$ at $T = 293$ K), c_0 the bulk concentration of KCl in the outer phase, and ϵ_0, R, T and F have their usual meaning.

In the case of the Donnan model it results in:

$$|\Delta\psi_D| = \frac{RT}{F} \ln \frac{A_l^- + \sqrt{(A_l^-)^2 + 4c_0^2}}{A_d^- + \sqrt{(A_d^-)^2 + 4c_0^2}} \qquad (7)$$

The values for σ_d and A_d^-, respectively, in Eqns. 6 and 7, are taken from the literature, namely $\sigma_d \approx 5 \cdot 10^{-4}$ $e^-/Å^2$ [6,17], based on a surface area of S/Chl ≈ 200 $Å^2$/Chl [18], and for $A_d = 30$ mM based on a volume of V_{in}/Chl ≈ 10 l/mol Chl [19] (see also Discussion).

Membrane conductance for nonactin-K^+ as a function of internal H^+ uptake

A change in the membrane conductance for the ionophore complex, nonactin-K^+, reflects a change in the potential, $\Delta\psi_s$, at the inner membrane surface (see Eqn. 5). The potential at the inner membrane

surface has been altered through changes of the H^+ concentration in the inner thylakoid space from pH_{in} 8 to 5 by preillumination with light of various intensities. This change corresponds to an H^+ uptake between 0 and 0.3 H^+/Chl. The H^+ and KCl concentrations of the outer phase were kept constant at pH_{out} 8 and $c_0 = 10$ mM. The result is shown in Fig. 6. Between pH_{in} 8 and 5 a relative change in the conductance for nonactin-K^+ from 1 to 3.8 was found:

$$\frac{G_1(pH_{in} \approx 8)}{G_2(pH_{in} \approx 5)} = 3.8 \qquad (8)$$

According to Eqn. 5 this change corresponds to an alteration of the K^+_{in} concentration by a factor of 3.8 and to a change of the membrane potential at the inner surface of $\Delta\psi_s = 34$ mV. Additionally, in Fig. 6 the expected Donnan potential difference, $\Delta\psi_D$ (Eqn. 7) and the Gouy-Chapman potential difference, $\Delta\psi_G$ (Eqn. 6) as a function of pH_{in} and ΔH^+/Chl, respectively, are indicated, taking the

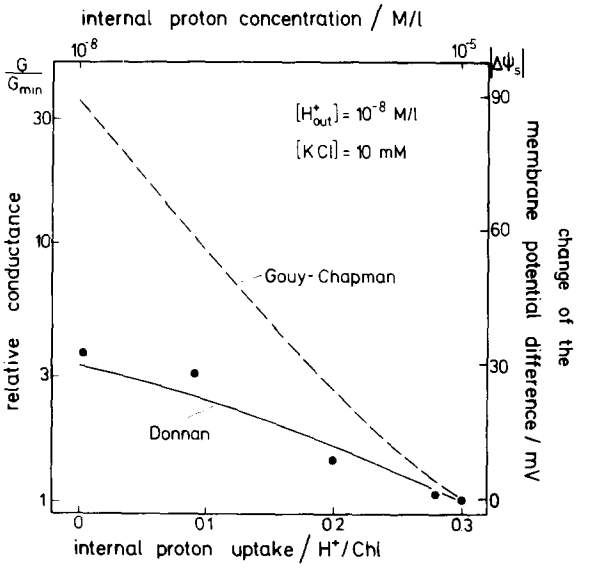

Fig. 6. Relative membrane conductance for nonactin-K^+ (0.3 μM) as a function of the internal H^+ uptake and the internal H^+ concentration, respectively (pH_{out} 8), at an outer bulk concentration of 10 mM KCl. The curves are calculated according to the Gouy-Chapman (- - - - -) and the Donnan (———) theories. $\Delta\psi_s$ is the difference of the inner surface membrane potential between ψ_{s1} (at conductivity G_1) and ψ_{s2} (at conductivity G_2) (see Eqn. 5).

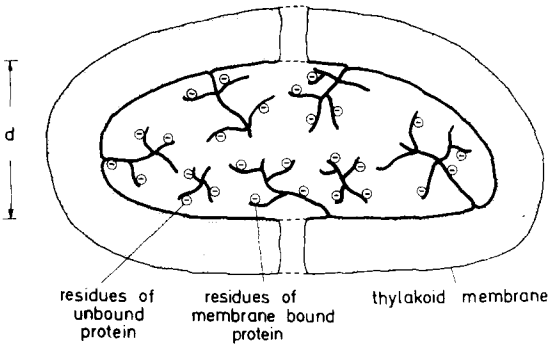

Fig. 7. Schematic depiction of the assumed distribution of membrane-bound and unbound proteins in the inner thylakoid space. The buffer groups of these proteins are indicated. The bar indicates the distance, d, between opposite membrane surfaces. Assuming an internal volume of 10 l/mol Chl it results in $d \approx 250$ Å for thylakoids shaped like oblate spheroids.

data outlined above. Again, the Donnan but not the Gouy-Chapman model provides a good fit for the measured dependence. The maximal change $\Delta\psi_S$ in Fig. 6, evaluated from the change of conductance (34 mV between pH_{in} 8 and 5 at $c_0 = 10$ mM) corresponds to the change of the transmembrane steady-state potential difference, $\Delta\psi$, at $c_0 =$ 10 mM in Fig. 5.

Discussion

We have measured the steady-state change of the transmembrane voltage, $\Delta\psi$, induced by saturating continuous light which shifts pH_{in} from 8 to 5. $\Delta\psi$ is strongly dependent on the KCl concentration, c_0, in the outer bulk phase. On varying the concentration of KCl from 1 to 100 mM, a monotonic decrease in the steady-state potential change from 80 to about 0 mV was found (Fig. 5, top). The H^+ uptake under these conditions is, independent of the salt concentration, 0.3 H^+/Chl (Fig. 5, bottom). A reasonable fit for the measured potentials, $\Delta\psi$(KCl), can be obtained by a calculation based on the Donnan model (solid line). This means that the H^+ is bound by buffer, A^-, distributed within the inner volume. Independent of the steady-state measurements, the conductance of nonactin-K^+, $G_{\text{nonactin-}K^+}$, as a function of pH_{in} at $c_0 =$ constant has been measured. In this case as well the Donnan model is in agreement with the measured

dependence $G(pH_{in})$. It is open to discussion whether the results in Figs. 5 and 6 can be explained also by a Gouy-Chapman model where the translocated H^+ is bound by buffer located at the inner surface of the membrane. However, with the parameters used (see Results and below) such a binding would lead to potentials and conductances much greater than the measured ones (dashed line in Figs. 5 and 6).

First, our experimental results should be compared with experimental data given in the literature. Then we have to discuss the validity of the data used for the calculation in the Donnan and Gouy-Chapman case.

With respect to particular potential values – 10 mV at 30 mM KCl and 80 mV at 1 mM KCl (see Fig. 5) – these are supported by results obtained by other methods. The former (10 mV) is in accordance with reports in Refs.4, 5 and 20. The latter (80 mV) is comparable with the results of Barber [21] based on the delayed light emission from spinach chloroplasts at 2 mM KCl. Gräber and Witt [22] 'titrated' with flashing light in whole *Chlorella* cells the steady-state potential and measured a value of 100 mV. However, in this case the ion strength in *Chlorella* cells was unknown, in contrast to isolated chloroplasts.

Potential measurements through electrochromic absorption changes were performed in our institute later on by Siggel [23] and Huber et al. [24]; however, with (a) chloroplasts which had previously been stored in a frozen state instead of freshly prepared chloroplasts, and (b) with negatively charged electron acceptors instead of positive ones. Under these conditions, their results in the concentration range greater than 3–5 mM KCl are similar to those reported in Ref. 7 and in this work; but, in the low salt range (3–5 mM KCl) significant deviations are noticed. With respect to the H^+ uptake, under such conditions, the dependence of the H^+ uptake on the salt content is the same (0.3 H^+/Chl) in the range 10–100 mM KCl, but much smaller between 1 and 10 mM KCl.

However, it was verified [25] that this difference is partly attributable to the application of differently charged electron acceptors, namely of the negatively charged anthraquinone 2-sulfate used by Siggel [23[and of the positively charged benzyl viologen used in this study. The pronounced salt dependence of the H^+ uptake that is monitored when anthraquinone 2-sulfate is applied can be explained by the repulsion of the anthraquinone 2-sulfate ions from the negatively charged external surface of the thylakoid membrane. This effect decreases the acceptor concentration of the donor side of the membrane, whereby the electron-transfer rate is diminished, resulting in a decrease in H^+ uptake. The repulsive force decreases with decreasing surface potential, i.e., by increasing the KCl concentration. Thereby electron transfer and H^+ uptake are fully restored. When the divalent positively charged benzyl viologen is applied, such complications do not occur. The use of chloroplasts which had been previously stored in a frozen state [23] instead of freshly prepared thylakoids (this study) with their different permeabilities might also be responsible for the H^+-uptake difference. With respect to the differentiation between the relevance of the Donnan or Gouy-Chapman model, one has to discusss the soundness of the values used for the calculation in both models. In the Donnan calculations we used for the concentration of the buffer groups, A_d^-, which are in this case assumed to be located in the inner thylakoid space, and $A_d^- = 30$ mM, respectively, on the basis of $V_{in} = 10$ l/Chl and $A_d^- = 0.3$ per Chl. The concentration chosen is quite realistic because in chloroplasts, quantities between $A^- =$ 0.2, and 0.6 Chl have been reported [19,36].

With the fit of the Donnan model an internal volume of 10 l/mol Chl is assumed. Such values are measured in the presence of 20–30 mM KCl for freshly prepared spinach chloroplasts with the phosphate method developed by Reinwald [19]. However, as reported in the literature [4], the internal volume increases with decreasing concentrations of permeable ions. Considering this, the calculated potential changes in the range of 10 mM KCl are not dramatically smaller (by 20 mV) than the measured ones. In the Gouy-Chapman calculation for the density, σ_d, of the buffer groups, A_d^-, which in this case are assumed to be located in the inner membrane surface, a value of $A_d^- = 0.1$ Chl has been used [17]. This results with a surface area of $S/\text{Chl} = 200$ Å^2/Chl [18] in $\sigma_d = 5 \cdot 10^{-4}$ e^-/Å^2. But, even if the highest value reported, namely, $A_d^- = 0.4$ Chl [26[which corresponds to $\sigma_d = 20 \cdot 10^{-4}$ e^-/Å^2 is regarded, the steady-state

potential calculated according to the Gouy-Chapman theory is, at KCl concentrations above 10 mM, by 20–30 mV higher than the measured values and below 10 mM up to 30 mV lower than the measured ones. Furthermore, Itoh [17] measured a change in the inner, negative surface charge density from $\sigma_d(pH_{in}\ 8) = 5 \cdot 10^{-4}\ e^-/Å^2$ to $\sigma_1(pH_{in}\ 5) = 3.5 \cdot 10^{-4}\ e^-/Å^2$. This small change indicates that the surface charges are not sufficient to account for the binding of H^+ actually pumped into the inner thylakoid space (0.3 H^+/Chl) which should lead to a positive value of $10 \cdot 10^{-4}\ e^-/Å^2$, i.e., under these circumstances even a charge reloading of the membrane surface to positive values should occur. This is inconsistent with the finding that the isoelectric point of the inner membrane surface is at pH ≈ 4.1 [27].

In contrast to Itoh [17], Huber and Rumberg [28] measured with the change of pH_{in} from 8 to 5 a decrease in the charge density from $17 \cdot 10^{-4}$ $e^-/Å^2$ to about zero. Huber et al. [24,28] assumed that the charge-bearing groups are connected with the membrane but are projecting into the inner thylakoid space up to a distance of 1.5 nm from the plane of the membrane. With this assumption they described the measured salt dependence of the steady-state potential difference reported in Refs. 24 and 25. This was done fairly well by means of a modified surface potential theory. This theory predicts the generation of a Donnan-like potential in the charged layer and a Gouy-Chapman-like potential outside the layer. However, the predicted thickness of 1.5 nm of this layer is a formal one and may not be identical with the real value. A layer of greater thickness but smaller charge density may also fit the experimental data. Electron microscopic photographs of the inner surface of the thylakoid membrane show a dense population of membrane-bound particles [29,30]. These particles are proteins; their hydrophobic part is embedded in the membrane, the hydrophilic part projects up to 20 nm into the inner thylakoid phase. As proteins are assumed to act as buffers [31], the charged buffer groups should also project up to 20 nm into the inner phase. A possible distribution of these membrane-bound buffer groups within the inner thylakoid volume is schematically depicted in Fig. 7. In as much as the minor axis of the thylakoids is smaller than 40 nm (V_{in}/Chl < 20 l/mol Chl) one can estimate that on average, the buffer groups are distributed over the whole thylakoid volume, where they should act as Donnan buffers in accordance with our conclusions. Furthermore, there are some hints that not only membrane-bound proteins are distributed in the inner thylakoid phase but also freely diffusible proteins such as plastocyanin [32]. A possible indication for the distribution of the buffer groups within the whole inner thylakoid phase might be also the observation that in subchloroplasts, a very low H^+ uptake creates an internal pH gradient similar to that in chloroplasts [33–35]. This can be explained by assuming that an essential amount of the buffer groups is washed out during the preparation. This is possible if the buffer groups project for into the inner space, thereby being loosely bound to the membrane, or if they are not connected to the membrane at all. A distribution of buffer groups within the inner thylakoid phase has also been concluded from H^+ flux measurements [36].

In summary, it can be stated that the observed strong dependence of the potential difference change on the ion concentration has to be taken into account when discussing the apparent differences in the values of the potential changes reported in the literature. In most cases the ion concentrations are not considered at all. Furthermore, the quantitative dependence (a) of the light-induced change of the electric potential difference on the ion concentration and (b) the dependence of the membrane conductivity for nonactin-K^+ on the H^+ concentration can be described by a Donnan model, i.e., by a distribution of buffer groups over the whole inner thylakoid space. Based on our experimental conditions (with respect to the inner thylakoid volume), this is realized if the membrane proteins project up to 20 nm into the inner space. The latter is in accordance with information obtained from electron microscopic photographs.

Acknowledgements

We wish to thank Drs. P. Gräber and U. Siggel for critical reading of the manuscript, Ms. D. DiFiore for valuable technical assistance and Ms. A. Schulze for the drawings. This work has been

financially supported by the Deutsche Forschungsgemeinschaft.

References

1 Junge, W. and Witt, H.T. (1968) Z. Naturforsch. 23b, 244–254
2 Witt, H.T. (1979) Biochim. Biopys. Acta 505, 355–430
3 Rumberg, B. and Siggel, U. (1969) Naturwissenschaften 56, 130–132
4 Rotternberg, H., Grunwald, T. and Avron, M. (1972) Eur. J. Biochem. 25, 54–63
5 Schröder, H., Muhle, H. and Rumberg, B. (1972) in Proceedings of the 2nd International Congress on Photosynthesis (Forti, G., Avron, M. and Melandri, A., eds.), pp. 919–930, Junk, The Hague
6 Rumberg, B. and Muhle, H. (1976) J. Bioelectrochem. Bioenerg. 3, 393–403
7 Tiemann, R., DiFiore, D. and Witt, H.T. (1979) in Abstracts of Poster Presentations, Annual Meeting of the Deutsche Gesellschaft für Biophysik (Adam, G. and Stark, G., eds.), p. 70, Springer, Berlin
8 Winget, G.D., Izawa, S. and Good, N.E. (1965) Biochem. Biophys. Res. Commun. 22, 438–443
9 Rüppel, H. and Witt, H.T. (1969) Methods Enzymol. 16, 316–380
10 Thorne, S.W., Horvath, G., Kahn, A. and Boardman, K. (1975) Proc. Natl. Acad. Sci. U.S.A. 72, 3858–3862
11 Garab. G.I., Paillotin, G. and Joliot, P. (1979) Biochim. Biophys. Acta 545, 445–453
12 McLaughlin, S.G.A., Szabo, G., Eisenmann, G. and Ciani, S.M. (1970) Proc. Natl. Acad. Sci. U.S.A. 67, 1268–1275
13 Gräber, P. and Witt, H.T. (1976) Biochim. Biophys. Acta 423, 141–163
14 Junge, W. and Ausländer, W. (1973) Biochim. Biophys. Acta 333, 59–70
15 Jones, M.N. (1975) Biological Interfaces, pp. 135–162, Elsevier, Amsterdam
16 Sten-Knudsen, O. (1978) in Membrane Transport in Biology (Giebisch, G., Testeson, D.C. and Ussing, H.H., eds.), Vol. 1, pp. 5–113, Springer, Berlin
17 Itoh, S. (1979) Biochim. Biophys. Acta 548, 579–595
18 Kirk, J.T.O. (1971) Annu. Rev. Biochem. 40, 161–196
19 Reinwald, E. (1970) Thesis, Technische Universität Berlin
20 Vredenberg, W.J. and Tork, W.J.M. (1975) Biochim. Biophys. Acta 387, 580–587
21 Barber, J. (1972) FEBS Lett. 20, 251–254
22 Gräber, P. and Witt, H.T. (1974) Biochim. Biophys. Acta 333, 389–392
23 Siggel, U. (1981) J. Bioelectrochem. Bioenerg. 8, 347–354
24 Huber, H.-L., Rumberg, B. and Siggel, U. (1980) Ber. Bunsenges. Phys. Chem. 84, 1050–1055
25 Siggel, U. (1981) in Proceedings of the 5th International Congress on Photosynthesis, Kallithea, Greece (Akoyunoglou, G., ed.), pp. 431–444, Balaban International Science Services, Philadelphia, PA
26 Yerkes, C.T. and Babcock, G.T. (1981) Biochim. Biophys. Acta 634, 19–29
27 Akerlund, H.-E., Andersson, B., Persson, A. and Albertsson, P.A. (1979) Biochim. Biophys. Acta 552, 238–246
28 Huber, H.-L. and Rumberg, B. (1981) in Proceedings of the 5th International Congress on Photosynthesis, Kallithea, Greece (Akoyunoglou, G., ed.), pp. 419–429, Balaban International Science Services, Philadelphia, PA
29 Miller, K.R. (1979) Spektrum Wiss. 12, 37–45
30 Andersson, B., Simpson, D.J. and Hoyer-Hansen, G. (1978) Carlsberg Res. Commun. 43, 77–89
31 Nakatani, H.Y., Barber, J. and Forrester, J.A. (1978) Biochim. Biophys. Acta 504, 215–225
32 Lockau, W. (1979) Eur. J. Biochem. 94, 365–373
33 Rottenberg, H. and Grunwald, T. (1972) Eur. J. Biochem. 25, 71–74
34 McCarthy, R.E. (1968) Biochem. Biophys. Res. Commun. 32, 37–43
35 Nelson, N., Drechsler, Z. and Neumann, J. (1970) J. Biol. Chem. 245, 143–151
36 Chow, W.S. and Hope, A.B. (1976) Austr. J. Plant Physiol. 3, 141–152
37 McLaughlin. S. (1977) Curr. Top. Membranes Transp. 9, 71–144
38 Junge, w. and Schmid, R. (1971) J. Membrane Biol. 4, 179–192
39 Schmid, R. and Junge, W. (1975) Biochim. Biophys. Acta 394, 76–92