Biochimica et Biophysica Acta, 357 (1974) 285–298

BBA 46783

THE ELECTRIC GENERATOR IN THE PHOTOSYNTHESIS OF GREEN PLANTS

II. KINETIC CORRELATION BETWEEN PROTOLYTIC REACTIONS AND REDOX REACTIONS

WINFRIED AUSLÄNDER and WOLFGANG JUNGE

Max-Volmer-Institut für Physikalische Chemie und Molekularbiologie, Technische Universität Berlin, D1 Berlin 12, Str. 17. Juni 135 (Berlin)

(Received February 4th, 1974)

SUMMARY

In a preceding paper (Junge, W. and Ausländer, W. (1974) Biochim. Biophys. Acta 333, 59–70), we attributed the four protolytic reactions at the outer and the inner side of the functional membrane of photosynthesis to the protolytic properties of the redox components, water, plastoquinone and the terminal acceptor. The experimental evidence presented was conclusive except for one argument. The rate of the protolytic reactions as detected by the dye cresol red after a short flash of light was considerably slower than the rate of the corresponding redox reactions.

In this communication it is demonstrated that the rate of proton uptake from the outer phase of the functional membrane is slowed down by a diffusion barrier for protons which shields the redox reaction sites at the outer side of the membrane against the outer aqueous phase. This barrier can be lowered by sand grinding the chloroplasts, by digitonin treatment and by uncoupling agents. At the extreme the barrier can be practically eliminated to yield rates of proton uptake matching the rates of the corresponding redox reactions. This gives conclusive evidence that the electrochemical potential difference which light induces across the functional membrane of photosynthesis is generated by a vectorial electron–hydrogen transport system as postulated by Mitchell (e.g. (1966) Biol. Rev. 41, 445–502).

INTRODUCTION

In the preceding paper [2] we discussed the mechanism of the electrochemical generator in the functional membrane of the photosynthesis of green plants. Four protolytic reaction sites of the functional membrane were identified, two of proton uptake from the outer phase [1, 2] and two of proton release into the inner one [1].

Abbreviations: DCIP, 2,6-dichlorophenol-indophenol; PMS, *N*-methylphenazonium methosulphate; FCCP, carbonylcyanide-*p*-trifluormethoxyphenylhydrazone.

The stoichiometry of these sites in relation to the number of electrons transferred from water to the terminal acceptor benzylviologen was within an experimental error of 10 % [1]. From the above stoichiometries and the deactivation of the redox and protolytic reaction sites we concluded that the four protolytic reactions observed are a direct consequence of the following redox reactions: the oxidation of water at the inner side of the functional membrane by Light Reaction II, the reduction of plastoquinone by Light Reaction II at the outer side, its oxidation at the inner one by Light Reaction I and the reduction of the terminal electron acceptor at the outer side of the membrane by Light Reaction I, respectively (see Fig. 5 in ref. 1). These results together with the finding that both light reactions contribute to the electric potential generation [2] we considered to be almost conclusive evidence for an electrochemical potential generation by vectorial electron-hydrogen transport as postulated by Mitchell [3, 4].

However, one experimental result might shed doubt on the above attribution of protolytic reactions to certain redox reactions, namely, the considerably slower rate of the protolytic reactions in comparison with the rate of the corresponding redox reactions. Since protolytic reactions in an aqueous environment are very rapid [5] one would expect that e.g. proton binding from the outer aqueous phase would follow the kinetics of the corresponding redox reactions at the outer side of the membrane (ms range) almost instantaneously. A similar discrepancy between the rates of the redox and the protolytic reactions in chromatophores has led to the suggestion that both are only indirectly linked to each other via a membrane Bohr effect [6]. If so, the above concept of the electrochemical generator has to be reevaluated.

We have demonstrated earlier, that the sand grinding of chloroplasts leads to an acceleration of the rate of proton binding from the outer phase [2]. This led us to propose that a diffusion barrier for protons shields the redox reaction sites at the outer side of the membrane against the outer aqueous phase. In this communication we demonstrate that this barrier is also lowered by detergent treatment and by proton permeability increasing agents. At the extreme this barrier can be practically eliminated so as to yield rates of proton binding matching the rates of the respective redox reactions.

Thus there are two types of arguments against a membrane Bohr effect in the functional membrane of the photosynthesis of green plants: the 1:1 stoichiometry between electrons and protons and their kinetic fit. All our experiments favoured an electrochemical generator for the proton which operates by a vectorial electron-hydrogen transport system in the membrane, as postulated by Mitchell [3, 4]. The chemical and structural nature of the permeability barrier for protons which shields the redox reaction sites from the outer aqueous phase has still to be elucidated. The results obtained so far lead us to postulate that this barrier covers a proton storage capacity which rapidly supplies protons to the redox reaction sites, being refilled slowly from the outer aqueous phase.

EXPERIMENTAL

Chloroplasts

Three types of chloroplasts were used in the experiments. After their prepara-

tion from market spinach the samples were stored under liquid N_2 until use. Thawed samples were suspended at an average chlorophyll concentration of 10 μM in the following standard reaction medium: KCl, 20 mM; $MgCl_2$, 2 mM; cresol red, 30 μM. Electron acceptors, uncoupling agents and buffer was added as indicated in the legends. Control chloroplasts were prepared according to the method specified in ref. 7. These chloroplasts belong to the broken type. Due to the hypoosmolar suspension they were highly swollen in the reaction medium.

Digitonin chloroplasts were prepared from a fresh preparation of control chloroplasts as follows: Control chloroplasts were suspended at an average chlorophyll concentration of 1 mg/ml in a medium containing tricine, 20 mM (pH 7.4); NaCl, 10 mM; $MgCl_2$, 1 mM. The temperature was kept at 2 °C. Digitonin was added with stirring to 0.5 %. After incubation for 30 min the suspension was centrifuged at $10000 \times g$ for 30 min. The supernatant was dialyzed for 30 min against a medium containing: sucrose, 0.4 M; tricine, 1 mM (pH 8); NaCl, 10 mM; $MgCl_2$, 1 mM; and dimethylsulphoxide, 5 %. After equilibration the suspension was stored under liquid nitrogen until use.

Ground chloroplasts were prepared from a fresh preparation of control chloroplasts as follows: 10 ml of chloroplasts with an average chlorophyll content of 1–2 mg/ml were ground for 15 min with 100 g sea sand at a temperature of 2 °C. Chloroplasts were extracted from the sand by washing with the dialyzing medium from above. Residual sand was removed from the suspension by centrifugation at low speed. Then the chloroplasts were sedimented at $5000 \times g$ and resuspended at an average chlorophyll concentration of 1 mg/ml in a medium identical with the above suspension medium for control chloroplasts (see under digitonin chloroplasts). The suspension was stored under liquid N_2 until use.

Excitation and record of transient absorption changes

Chloroplast suspensions were filled into a 2-cm absorption cell which was mounted in a rapid kinetic spectrophotometer. Photosynthesis was stimulated by excitation with a "single turnover flash". Transient absorption changes were recorded. The signal to noise ratio was improved by averaging over several transients induced by repetitive flashes. The repetition rate is indicated in the legends. For details and for specific references see our preceding paper [1].

Electron transport and light reaction activity

The number of electrons transferred by Light Reaction I was monitored via the rapid negative directed absorption changes of 2,6-dichlorophenol-indophenol (DCIP) at a wavelength of 574 nm following the studies of Kok [8]. The molar extinction coefficient at this wavelength is $2 \cdot 10^4$ [9]. The number of electrons supplied by water via Light Reaction II was monitored by the irreversible component of the negative directed absorption changes of DCIP at 574 nm [8]. Although not fully quantitative these experiments yielded an insight into the ratio of activity of the two light reactions.

Protolytic reactions

pH changes in the outer aqueous phase of the inner chloroplast membrane were measured with the pH-indicating dye cresol red at a pH around 8. Check-up

procedures and calibrations were discussed in our preceding paper [1]. The pH-indicating "absorption changes of cresol red at 574 nm" which are represented in the figures were measured as follows. Firstly the absorption changes at 574 nm were measured for a chloroplast suspension without buffer (standard reaction medium), the repetitive signals were summed in the averaging computer (CAT 1000) and then the same number of signals was subtracted obtained from chloroplasts in the presence of buffer (tricine, 1 mM (pH8)). The resulting signal difference represents the response of cresol red to pH changes while the contribution of the intrinsic absorption changes of chloroplasts is eliminated. The absence of aging during the sampling period was checked by comparison of the absorption changes resulting from sampling periods of doubled and halved length, respectively.

In the figures the ordinate label "different scale factors" indicated that we used a different number of repetitions to obtain the documented signals. This was for convenience only, to represent signals comparable in extent and signal to the noise ratio. There are several reasons for variations in the extent of the pH-indicating absorption changes in our experiments: (1) Digitonin chloroplasts and ground chloroplasts possess a greater buffer capacity than control chloroplasts. The structural reasons for this will be discussed in a subsequent paper. (2) Digitonin and grinding reduce the activity ratio of Light Reaction II over Light reaction I (for digitonin, see ref. 10). We studied the protolytic reactions in treated chloroplasts which are enriched in Photosystem I with different electron acceptors. It is evident that proton binding from the outer phase at Light Reaction I will be to a different extent depending on whether benzylviologen (non-cyclic) or DCIP (cyclic electron transport) is used as acceptor. In the first case the reaction is limited by the small number of active Light Reaction II centres, which survived the treatment, while in the latter it is not. (3) In the presence of ferricyanide, proton uptake from the outer phase is due to Light Reaction II, only. (4) Uncouplers contribute to the buffer capacity of chloroplast suspensions.
The frequency of the repetitive flashes was chosen to allow for the relaxation of the light-induced pH difference across the membrane. Since the treatment of chloroplasts by grinding or digitonin accelerated the relaxation rate the repetition rate was increased up to 0.5 s^{-1} for treated chloroplasts in comparison with 0.1 s^{-1} for the control.

RESULTS

Factors affecting the kinetics of proton binding from the outer phase

Grinding and treatment with digitonin. It is obvious from a comparison of the three traces in the left of Fig. 1 that grinding and treatment with digitonin accelerates the rate of proton binding after a short flash of light from the outer aqueous phase. The rise of absorption of the absorption changes of cresol red at 574 nm indicates alkalisation of the outer phase [1]. This suggests that the rate of proton uptake from the outer phase is determined by the gross structure of the inner membrane of chloroplasts. (It is worth mentioning that even the control chloroplasts lacked an intact outer membrane.)

It is also obvious that from a comparison of the traces in the left and in the right of Fig. 1, the rate of proton binding was furthermore accelerated if DCIP instead of benzylviologen was used as electron acceptor.

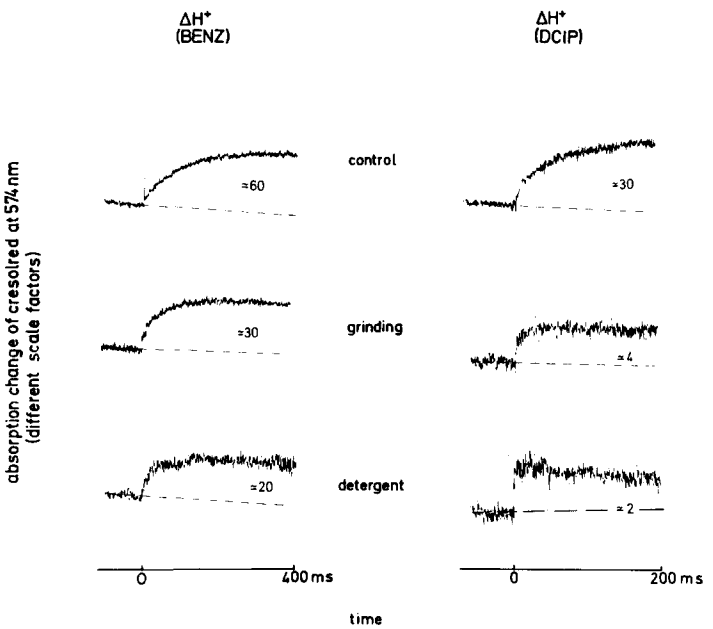

Fig. 1. Absorption change of cresol red at 574 nm induced by a single turnover flash of light at $t = 0$ (standard reaction medium). Left: benzylviologen (60 μM). Right: DCIP (10 μM). The numbers inserted into the traces indicate the approximate halftime of proton binding from the outer phase.

In the control chloroplasts both acceptors at their given concentration (benzylviologen, 60 μM; DCIP, 10 μM) yielded the same extent of the rapid negative directed absorption change of chlorophyll a_1 at 705 nm, which indicates that the Light Reaction I activity was practically unaltered (see ref. 1). The same extent of change was observed for the electrochroic absorption changes at 524 nm, which indicates that

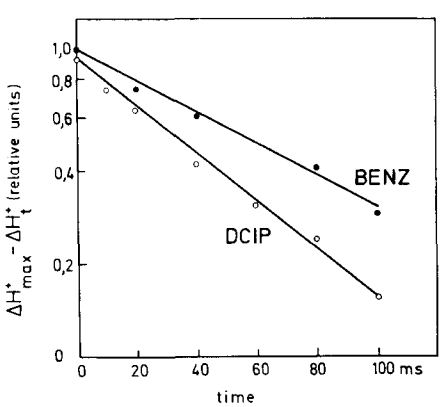

Fig. 2. Proton uptake kinetics of control chloroplasts with benzylviologen (BENZ) and DCIP, respectively (see Fig. 1, first row). The ordinate scale is the same for both electron acceptors.

the activity of both light reactions was unaltered when exchanging DCIP for benzylviologen as electron acceptor (see ref. 1). Thus, in control chloroplasts the acceleration of the proton binding by DCIP could not be understood by any alteration of the relative contribution of the two light reactions to proton binding from the outer phase. But then it is puzzling that kinetic analysis (see Fig. 2) yields a good approximation to a first-order process, although in control chloroplasts two different reaction sites (see ref. 1) contribute about equal amounts to proton binding only one of which is linked to the reduction of the terminal electron acceptor, which was exchanged in the experiments depicted in Figs 1 and 2. This suggests that the additional acceleration of proton binding by DCIP might be due to its uncoupling properties [11, 12] rather than its redox properties. So we studied the influence of different types of electron acceptors and of uncouplers.

Electron acceptors. Saha et al. [12] classified the acceptors of photosynthetic electron transport into three distinct categories: ionic or strongly polar substances with negligible lipid solubility were placed in Class I, Class II contains lipid-soluble weak acids which can serve both as electron acceptors and uncouplers, all nonionic lipid-soluble acceptors belong to Class III. The latter, under certain conditions, may accept electrons between the two light reactions.

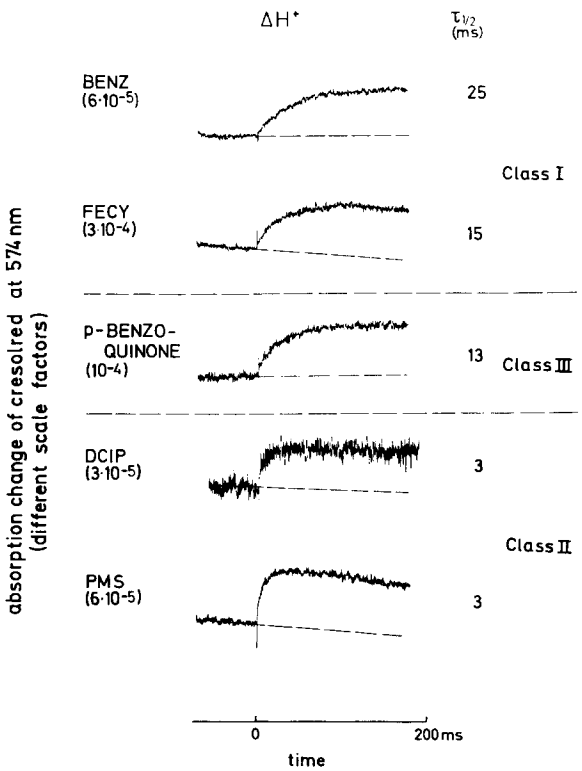

Fig. 3. Dependence of the proton uptake kinetics on different types of acceptors (standard reaction medium, ground chloroplasts). The molarity of the electron acceptors is given in parentheses. BENZ, benzylviologen; FECY, ferricyanide.

The kinetics of proton binding from the outer phase for ground chloroplasts with dependence on electron acceptors belonging to different classes is documented in Fig. 3. We find that electron acceptors belonging to Class II accelerate the rate of proton binding beyond the range of variation of this rate among different acceptors of Class I and Class III. Class II acceptors differ from the other ones by their uncoupler properties. So we tested whether uncoupling agents together with electron acceptors of Class I and III, respectively, yielded the same high rates as Class II acceptors.

Uncouplers. Fig. 4 shows the dependence of the kinetics of proton binding from the outer phase on the concentration of the uncoupler NH_4Cl (ground chloroplasts) in the presence of the Class I acceptor benzylviologen. As is obvious from Fig. 4 the rate of proton binding is accelerated with increasing uncoupler concentration. At the highest concentration (lower trace) the uncoupler acted not only on the kinetics of proton binding from the outer phase (halftime about 4 ms) but on the main permeability barrier which separates the inner aqueous phase from the outer one of the functional vesicles of photosynthesis in green plants. It increased the proton perme-

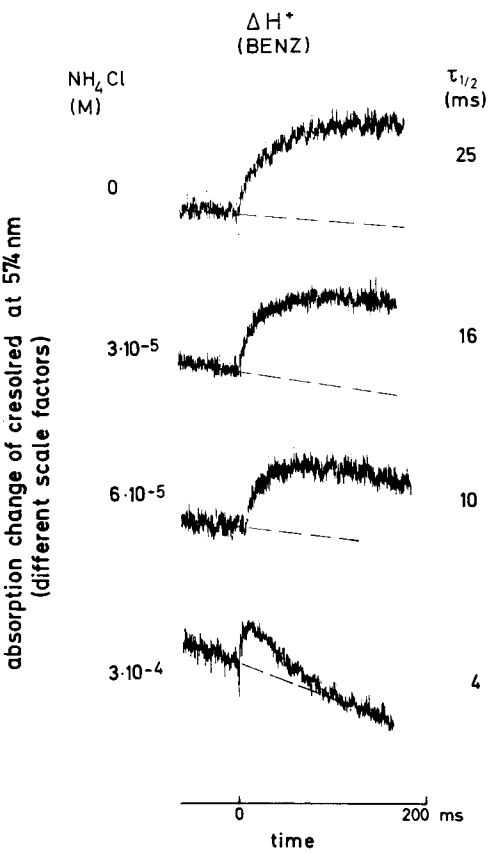

Fig. 4. Dependence of the proton uptake kinetics on the concentration of the uncoupler NH_4Cl (standard reaction medium, ground chloroplasts). Electron acceptor: benzylviologen (60 μM).

ability of the functional membrane so that the light-induced pH difference relaxed within 35 ms.

It has to be mentioned that the effect of the uncoupler NH_4Cl on the rate of proton binding is less pronounced for control chloroplasts than for ground chloroplasts. With benzylviologen as acceptor the halftime of the alkalisation was 60 ms (NH_4Cl, 0 μM), 27 ms (60 μM) and 10 ms (300 μM), respectively. This effect will be discussed in a subsequent paper.

The experimental results depicted in Fig. 4 reveal that Class I acceptor together with an uncoupling agent yields the same high rate of proton binding from the outer phase as Class II acceptors. This leads to the conclusion that the effect of Class II acceptors is due to their ability to act as uncouplers.

The experimental evidence presented so far suggests that a lipophilic diffusion barrier for protons shields the primary proton binding sites of the electron transport at the outer side of the functional membrane from the outer aqueous phase. The height of this barrier can be lowered by mechanical and chemical treatment of chloroplasts and by uncoupling agents.

Conditions for parallel kinetics of proton binding and redox reaction

We attributed proton binding from the outer phase of the functional vesicles to the reduction of plastoquinone and the terminal electron acceptor by Light Reactions I and II, respectively [1]. These redox reactions are rather rapid with time constants in the range of ms or even less. We learned that the observed delay of proton

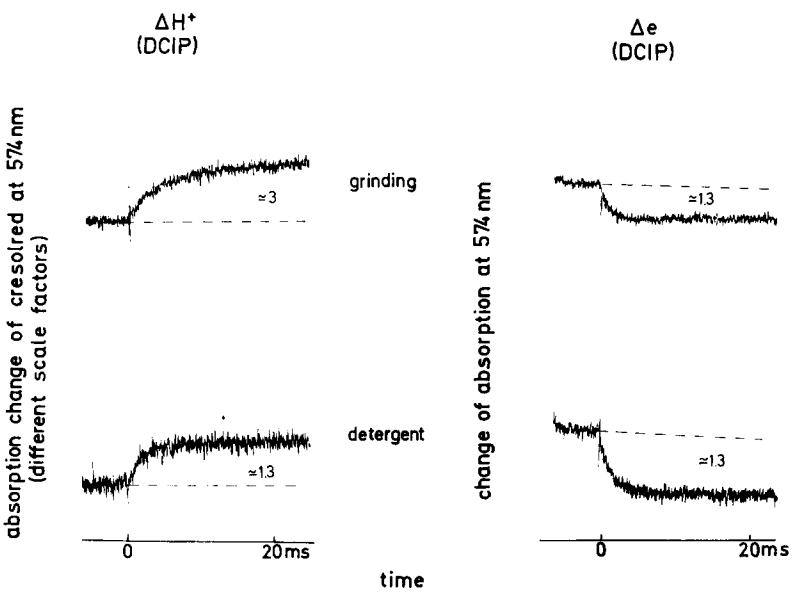

Fig. 5. Correlation of the kinetics of proton binding and DCIP reduction (DCIP, 10 μM). Upper traces: ground chloroplasts; Lower traces: digitonin-treated chloroplasts; Left traces: "absorption changes of cresol red at 574 nm" (see Experimental); standard reaction medium; Right traces: absorption changes of DCIP measured in the absence of cresol red and in the presence of buffer (tricine 1 mM, pH 8).

TABLE I

CORRELATION OF THE HALFTIME OF DCIP REDUCTION WITH THE HALFTIME OF PROTON UPTAKE AS DEPENDENT ON THE DCIP CONCENTRATION

DCIP (M)	Δe $\tau_{\frac{1}{2}}$(ms)	ΔH^+ $\tau_{\frac{1}{2}}$(ms)
$1.3 \cdot 10^{-6}$	5	7
$3 \cdot 10^{-6}$	2.2	2.5
10^{-5}	1.3	1.3
$3 \cdot 10^{-5}$	< 0.5	< 0.5

binding against these redox reactions might be due to a diffusion barrier for protons shielding the redox reaction sites from the outer aqueous phase. We ask whether this barrier can be lowered in order to eliminate the delay. In order to differentiate between the two proton binding sites, which are associated with different light reactions we carried out two sets of experiments each with only one of the light reactions contributing to the proton binding.

Digitonin-treated chloroplasts are enriched in Light Reaction I centres. This is known from the literature [10] and we checked it according to the method given in Experimental. We determined a Light Reaction I to Light Reaction II activity ratio of 4 (see Table II) which was paralleled by a H^+/e^- ratio of only 1.2 (the method for the determination of the latter is given in ref. 1). Thus proton binding from the outer aqueous phase is dominated by the reduction of the terminal acceptor by Light Reaction I in these chloroplasts.

The rate of DCIP reduction by Light Reaction I can be determined by monitoring the rapid negative directed absorption changes of this compound e.g. at 574 nm [8]. At the concentration (10 μM) and the redox state of DCIP we used in the experiments which are documented in Fig. 5 the halftime of the DCIP reduction was about 1.3 ms for ground chloroplasts, detergent-treated ones and for the control also. The halftime of proton binding from the outer phase under the same conditions was 30 ms (control), 3 ms (ground chloroplasts, Fig. 5) and about 1.3 ms (digitonin, Fig. 5), respectively. Thus at least in digitonin-treated chloroplasts the rate of proton binding at Light Reaction I followed the rate of the corresponding redox reaction without detectable delay. That the agreement of the rates is not accidental is demonstrated in

TABLE II

SUMMARY OF THE EXPERIMENTAL RESULTS WITH DIFFERENTLY TREATED CHLOROPLASTS

PSI/PSII: ratio of the activities of Light Reaction I and Light Reaction II. H/e^-(DCIP): proton uptake per electron transferred on excitation with a single turnover flash (electron transfer measured by the reduction of DCIP).

Type	ΔH^+(benzylviologen) $\tau_{\frac{1}{2}}$ (ms)	ΔH^+(DCIP) $\tau_{\frac{1}{2}}$ (ms)	Δe(DCIP) $\tau_{\frac{1}{2}}$ (ms)	PSI/PSII	$\frac{H^+}{e^-}$ (DCIP)
Control	60 ± 10	30 ± 5	1.3 ± 0.2	1	2 ± 0.2
Grinding	30 ± 5	3 ± 2	1.3 ± 0.2	1.5	1.5 ± 0.1
Detergent	20 ± 3	1.3 ± 0.2	1.3 ± 0.2	4	1.2 ± 0.1

Table I. Variation of the DCIP concentration over more than one order of magnitude leaves the rates of proton binding and of the DCIP reduction in reasonable agreement.

Next we studied the correlation between the rate of proton binding at Light Reaction II with the rate of the reduction of plastoquinone to which it was attributed in our preceding paper [1]. The halftime of the plastoquinone reduction was resolved by flash photometry. It amounts to 0.6 ms [13]. Proton binding from the outer phase in the presence of ferricyanide at low concentrations is due to Light Reaction II, only [1]. We accelerated the rate of proton binding at this site by adding the uncoupler carbonylcyanide-*p*-trifluoromethoxyphenylhydrazone (FCCP) in order to find out whether the rate of proton binding at Light Reaction II can be accelerated to match the rate of the plastoquinone reduction. The result, which is documented in Fig. 6 reveals a rapid proton binding with a halftime of about 2 ms for control chloroplasts with the acceptor ferricyanide (300 μM) and the uncoupler FCCP (1 μM). The time course of the pH-indicating absorption changes of cresol red at the low time resolution (left traces) was discussed in detail in our preceding paper [1].

We observed a halftime of alkalisation as short as 2 ms for control chloroplasts in the presence of FCCP, only, if ferricyanide was present, while the uncoupler was less effective in the presence of benzylviologen. The reasons for this synergistic effect of ferricyanide will be discussed in detail in a subsequent paper. The maximum rise time of the alkalisation of 2 ms in the presence of FCCP is still in disagreement with the 0.6-ms rise time of the plastoquinone reduction. This may be due to a residual proton-permeability barrier, resistant to the proton carrier FCCP [14], delaying proton binding against the plastoquinone reduction. However, preliminary evidence suggests that the 2 ms we observed for proton binding may reflect the true protonation kinetics of reduced plastoquinone, which then is delayed against reduction. Haehnel

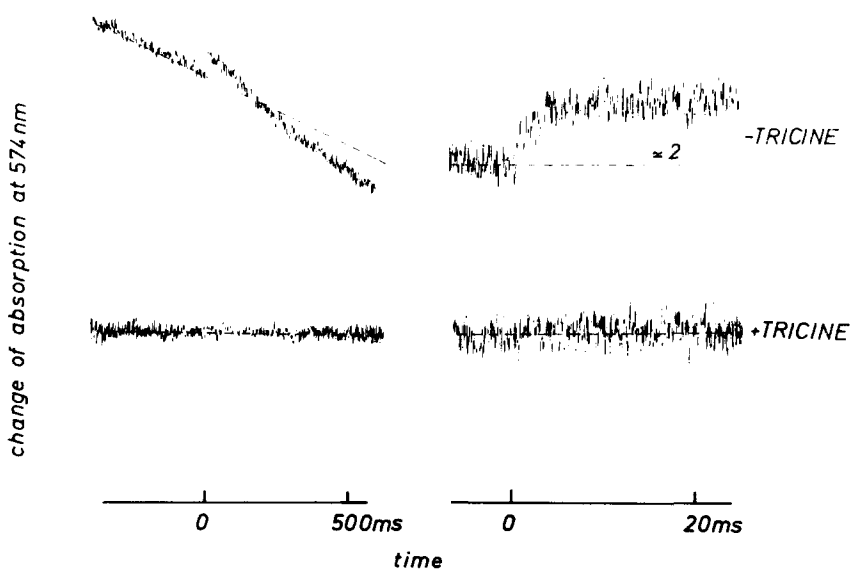

Fig. 6. Absorption changes at 574 nm with control chloroplasts with ferricyanide (300 μM) and the uncoupler FCCP (1 μM); standard reaction medium. Above: no buffer; below: with buffer (tricine, 1 mM, pH 8).

[15] reported a time lag of 2 ms for the reduction between plastoquinone and chlorophyll a_1. This time lag depends on the pH of the outer phase (Haehnel, personal communication).

Although the rates of proton binding and plastoquinone reduction could not be brought to a strict coincidence, the above experiments have demonstrated, that the discrepancy between these rates in untreated chloroplasts is due to a diffusion barrier for protons which can be overcome e.g. by proton carriers as FCCP.

DISCUSSION

The mechanism of the electric generator

In the preceding paper in this series [1] we presented evidence that the light-driven proton translocation across the inner chloroplast membrane is the direct consequence of vectorial electron-hydrogen transport between carriers at the inner and at the outer side of the functional membrane. These carriers undergo protolytic reactions with the neighbouring aqueous phase. Experiments on the proton electron ratios and on the deactivation of the redox and protolytic reaction sites confirmed the model for the electrochemical potential generation which is illustrated in the left of Fig. 7. The water-splitting system located at the inner side of the functional membrane, electron transport by Light Reaction II goes towards the outer side thus electrically charging the membrane. The reduction of plastoquinone at the outer side is followed by protonation, while its oxidation at the inner side leads to a proton release into the inner phase. So it acts as a carrier for hydrogen. Electron transport at Light Reaction I from the inner to the outer side then again contributes to the electric potential generation. This model was originally proposed by Mitchell [3, 4].

There was only one argument left against the attribution of the four protolytic reaction sites to the above electron carriers, namely the discrepancy between the low rates of the protolytic reactions and the higher rates of the redox reactions [6]. Now

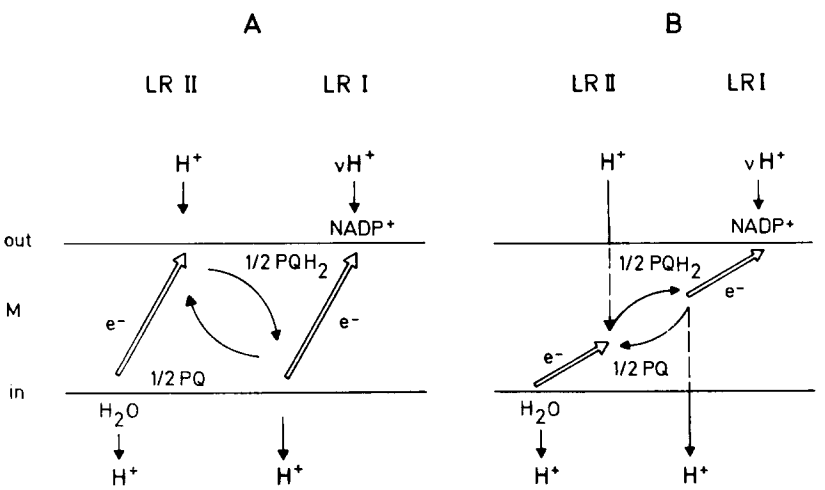

Fig. 7. Two models for the electric generator in the functional membrane of photosynthesis. LR, Light Reaction. PQ, plastoquinone.

this argument can be rejected based on the experimental evidence presented herein, which confirmed that the discrepancy between the reaction rates is due to a diffusion barrier shielding the redox reaction sites at the outer side of the membrane from the outer aqueous phase. Thus we can see no objection against a Mitchellian mechanism for the electrochemical generator in the functional membrane of photosynthesis of green plants.

However, as the attribution of certain redox reactions to the outer and the inner side of the functional membrane was mainly based on the access of protons from either aqueous phase to these sites [1], the vectorial electron transport scheme depicted in the left of Fig. 7 has to be contrasted with the modified version in the right. Common to both models is the contribution of both light reactions to the electrochemical potential generation which is equivalent to the translocation of two protons from the outer to the inner side of the major dielectric layer of the membrane. The alternative model (Fig. 7B) essentially differs from the original one (A), only, if the membrane space represents a more or less homogeneous dielectric. (The electric conductivity should be low so that any electric field in the membrane is not annihilated before a msecond.) But then not only the electron transport at both light reactions but the proton flux across the membrane (dotted arrows in Fig. 7B) would contribute to the electric potential generation. The electric potential which is indicated by the electrochroic absorption changes at 520 nm [16, 17] has a rise time of less than 20 ns [18]. Thus the alternative model for the electric potential generation (Fig. 7B) does not only require vectorial electron transport but in addition vectorial proton translocation at this extremely high velocity. For its higher degree of complexity we regard the alternative model as improbable.

The structure of the proton diffusion barrier at the outer side of the chloroplast inner membrane

The chemical and the structural nature of the proton diffusion barrier at the outer side of the functional membrane of photosynthesis has still to be elucidated. It is an open question whether the barrier is formed by isolated knobs covering each light reaction centre, or by a continuous lipophilic layer covering the whole membrane, or if the barrier is due to electrically poorly conducting material in the interthylakoid space of grana stacks. Moreover, we are as yet unable to decide whether the lipophilic space which forms the barrier is dominated by lipids or by protein. Let us assume, just for an illustration, that the proton permeability barrier is formed by a rather homogeneous surface layer shielding the dielectric core of the functional membrane from the outer aqueous phase. Then our results imply that the rapid electron transfer at both light reactions from a donor at the inner side to an acceptor at the outer side of the membrane ends beneath this hydrophobic surface layer, although it has crossed the main dielectric layer (the membrane core in Fig. 8). In our preceding paper we located electron carriers at either side of the membrane [1]. The location of an acceptor A "at the outer side" in operational definition then means: reduced A is more readily accessible to protons from the outer phase than to those from the inner one. The above experiments revealed that the halftime of access of protons from the outer phase to the reducing sites of both light reactions is about 60 ms (see Fig. 1, benzylviologen), while it takes several seconds for protons from the inner phase to leak into the outer one via the membrane core.

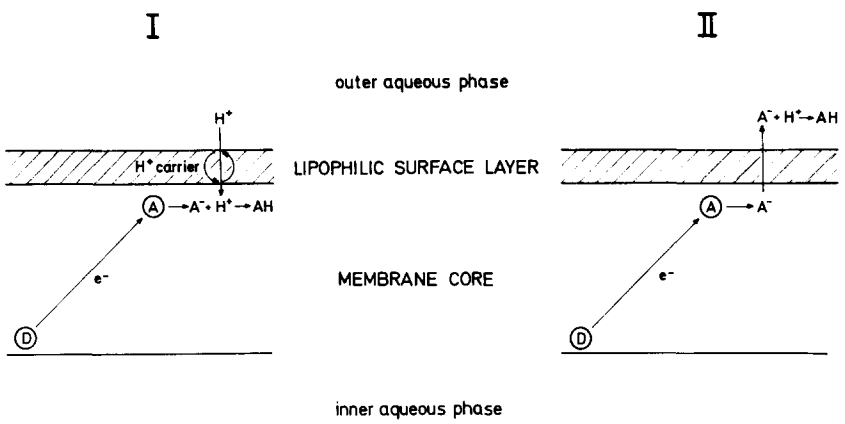

Fig. 8. Models for the observed delay between redox reactions and proton binding from the outer phase (details see text).

The protonation of the acceptor A beneath the lipophilic surface layer by protons from the outer phase may proceed via two alternative mechanisms which are illustrated in Fig. 8. Either the protons move across the lipophilic surface layer (Model I) or the reduced acceptor moves outward. In our experiments with Class II acceptors (Figs 1–3) and with the uncouplers NH_4Cl (Fig. 4) and FCCP (Fig. 6), all of them can act as proton carriers [11, 14, 19], and can be understood in terms of the first model (Fig. 8, left), only.

Our experiments on the proton binding in control chloroplasts in the presence of benzylviologen revealed a halftime of about 60 ms for both proton binding sites (Figs 1 and 2). One of these sites was attributed to the reduction of plastoquinone which has a halftime of 0.6 ms [13] while the subsequent reoxidation takes about 20 ms [20]. Thus the redox cycle of plastoquinone is turned over once before the outer aqueous phase receives the information thereafter via consecutive protolytic reactions. So we have to postulate the existence of a proton reservoir beneath the lipophilic surface layer, which rapidly supplies protons to the reduced electron acceptors and only slowly refills from the outer phase (under flash conditions). It is obvious that the refill rate under steady light (greater proton gradient) may increase.

Independent evidence supports the hypothesis of a proton storage capacity inside the membrane in addition to the well-known storage capacity of the inner aqueous phase of the functional vesicles. Nishizaki [21] reported two-phasic kinetics of the proton efflux from broken chloroplasts after an acid-base transition. He interpreted the rapid phase as due to proton release from within the membrane while the slower phase was attributed to proton efflux from the inner phase across the membrane.

The above experiments gave evidence for the existence of a diffusion barrier for protons at the outer side of the inner chloroplast membrane, which shield the reducing sites at both light reactions against the outer aqueous phase. A proton reservoir had to be postulated between this diffusion barrier and the membrane core. The relative contributions of proteins and lipids to these structural elements will be discussed in the subsequent communication.

ACKNOWLEDGEMENTS

We wish to thank Mrs I. Columbus for technical assistance.

This work has been supported by a grant from the Deutsche Forschungsgemeinschaft.

REFERENCES

1 Junge, W. and Ausländer, W. (1974) Biochim. Biophys. Acta 333, 59–70
2 Schliephake, W., Junge, W. and Witt, H. T. (1968) Z. Naturforsch. 23b, 1561–1578
3 Mitchell, P. (1961) Nature 191, 144–148
4 Mitchell, P. (1966) Biol. Rev. 41, 445–502
5 Eigen, M. (1965) Discuss. Faraday Soc. 39, 7–15
6 Chance, B., McCray, J. A. and Bunkenburg, J. (1970) Nature 225, 705–708
7 Siggel, U., Renger, G., Stiehl, H. H. and Rumberg, B. (1972) Biochim. Biophys. Acta 256, 328–335
8 Kok, B., Malkin, S., Owens, O. and Forbush, B. (1966) Brookhaven Symp. Biol. 19, 446–459
9 Punnet, T. (1959) Plant. Physiol. 34, 283–289
10 Anderson, J. M. and Boardman, N. K. (1966) Biochim. Biophys. Acta 112, 403–421
11 Elhanan, Z. G. and Avron, M. (1964) Biochemistry 3, 365–373
12 Saha, S., Ouitrakul, R., Izawa, S. and Good, N. E. (1971) J. Biol. Chem. 246, 3204–3209
13 Vater, J., Renger, G., Stiehl, H. H. and Witt, H. T. (1968) Naturwissenschaften 55, 220–221
14 Liberman, Ye. A. and Topaly, V. P. (1968) Biofizika (Transl.) 13, 1195–1207
15 Haehnel, W. (1973) Biochim. Biophys. Acta 305, 618–631
16 Junge, W. and Witt, H. T. (1968) Z. Naturforsch. 23b, 244–254
17 Emrich, H. M., Junge, W. and Witt, H. T. (1969) Z. Naturforsch. 24b, 1144–1146
18 Wolff, Chr., Buchwald, H. E., Rüppel, H., Witt, K. and Witt, H. T. (1969) Z. Naturforsch. 24b, 1038–1041
19 Crofts, A. R. (1967) J. Biol. Chem. 242, 3352–3359
20 Stiehl, H. H. and Witt, H. T. (1969) Z. Naturforsch. 24b, 1588–1599
21 Nishizaki, Y. (1972) Biochim. Biophys. Acta 275, 177–181