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DECAY OF MEMBRANE POTENTIAL UNDER PHOSPHORYLATING CONDITIONS IN CHLOROPLASTS WITH IN VIVO ACTIVATED ATPase

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(1) The relation between the membrane potential and phosphorylation was studied in chloroplasts rapidly prepared from illuminated spinach leaves (light chloroplasts) and from dark-adapted leaves (dark chloroplasts). Light chloroplasts had a higher ATP hydrolysis activity than dark chloroplasts. (2) In the presence of ADP or ATP, a rapidly decaying phase of the field-indicating 518 nm absorbance change with a half-time of 15 ms became apparent in addition to the slow phase with a half-time of more than 300 ms in either type of chloroplast. Under these conditions, light chloroplasts showed a larger rapid phase than dark chloroplasts. (3) The rapid phase was suppressed by dicyclohexylcarbodiimide and was assumed to reflect the dissipation of membrane potential due to proton movements inside the CF_1-CF_0 ATP synthetase. (4) A model for the proton movement in ATP synthetase is proposed.

Introduction

ATP synthesis from ADP and P_i in chloroplasts is driven by the electrochemical proton potential ($\Delta\tilde{\mu}_{H^+}$) formed across the thylakoid membrane according to the chemiosmotic hypothesis of Mitchell [1,2]. This was evidenced by the accelerated decay of the membrane potential due to potential-driven proton efflux via the reversible ATP synthetase (CF_1CF_0-ATPase) on the membrane, by the measurement of the field-indicating 518 nm absorbance change (ΔA_{518}) [3,4]. There seems to exist a threshold of $\Delta\tilde{\mu}_{H^+}$ for the activation of the ATP synthetase in ordinarily prepared class II chloroplasts [5]. The threshold seems to be tightly related to the mechanism of ATP synthesis.

The activity of ATP hydrolysis in isolated chloroplasts can be elevated if chloroplasts are rapidly prepared from intact leaves after prior illumination [6,7]. This activation seems to be the results of chemical modification of the CF_1 moiety of the ATP synthetase by the thioredoxin system [8]. The ATP hydrolysis activity of chloroplasts (light chloroplasts) rapidly isolated (within 90 s after the end of illumination) from illuminated leaves was more than 20-times higher than that of chloroplasts (dark chloroplasts) isolated from dark-adapted leaves [6]. It was also shown that the in vivo decay rate of ΔA_{518} induced by a single-turnover flash light was significantly accelerated in the preilluminated leaves [6,9]. the decay rate was also very fast in isolated light chloroplasts as far as the measurement was performed within a few minutes after the isolation of chloroplasts [6].

The in vivo acceleration of ΔA_{518} decay was suppressed by DCCD, an inhibitor of proton con-

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Abbreviations: dark and light chloroplasts, chloroplasts rapidly prepared (90 s for the preparation time) from illuminated and dark-adapted leaves, respectively; ΔA_{518}, absorption change at 518 nm; DCCD, *N*,*N'* dicyclohexylcarbodiimide; Tricine, *N*-tris(hydroxymethyl)methylglycine.

duction through the CF_0 moiety of ATP synthetase [6]. These results suggest that the increase in ATPase activity of ATP synthetase leads to rapid dissipation of the membrane potential due to the accelerated proton flow coupled to ATP synthesis [6]. Light chloroplasts actually show a higher ATP synthesis rate under some conditions, in which the turnover rate of ATP synthetase is the rate-limiting step, than dark chloroplasts (Morita, S., Itoh, S. and Nishimura, M., unpublished data). In the present study, ΔA_{518} was measured in dark and light chloroplasts and the relation between the rate of field decay and phosphorylation was studied.

Materials and Methods

Chloroplasts were prepared in a way essentially the same as that reported previously [6]. Dark-adapted leaf segments (incubated in darkness for more than 2 h) of market spinach were preilluminated by light from a 650 W slide projector through a Hoya HA-50 heat-absorbing filter ($1.8 \cdot 10^5$ erg $\cdot$ cm$^{-2} \cdot$ s^{-1} at the leaf surface) at room temperature (14–17°C), and then chloroplasts were rapidly isolated by the following procedure: leaf segments were rapidly homogenized by a Biotron homogenizer for 3 s in 9 ml of a medium containing 10 mM $MgCl_2$ and 50 mM Tricine-KOH (pH 8.0) buffer at about 0°C, and filtered through four layers of cheesecloth. 1.5 ml of the filtrate were centrifuged at $9000 \times g$ for 20 s in an Eppendorf Centrifuge 5412. The pellet obtained was suspended in a precooled medium containing 6 mM phosphate buffer (pH 8.0), 50 mM KCl and 10 mM $MgCl_2$ and again centrifuged at $9000 \times g$ for 30 s. The precipitates obtained after the centrifugation were dispersed in the same medium and stored on ice (light chloroplasts). The illumination step was omitted in the preparation of dark chloroplasts. The time required for the preparation of chloroplasts after the end of illumination was about 90 s. Most of the procedures were carried out under a dim green light. Each preparation of chloroplasts thus obtained was renewed after about 1 h (including the time required for the measurement).

The absorbance change at 518 nm (ΔA_{518}) was measured with a rapid split-beam spectrophotome-

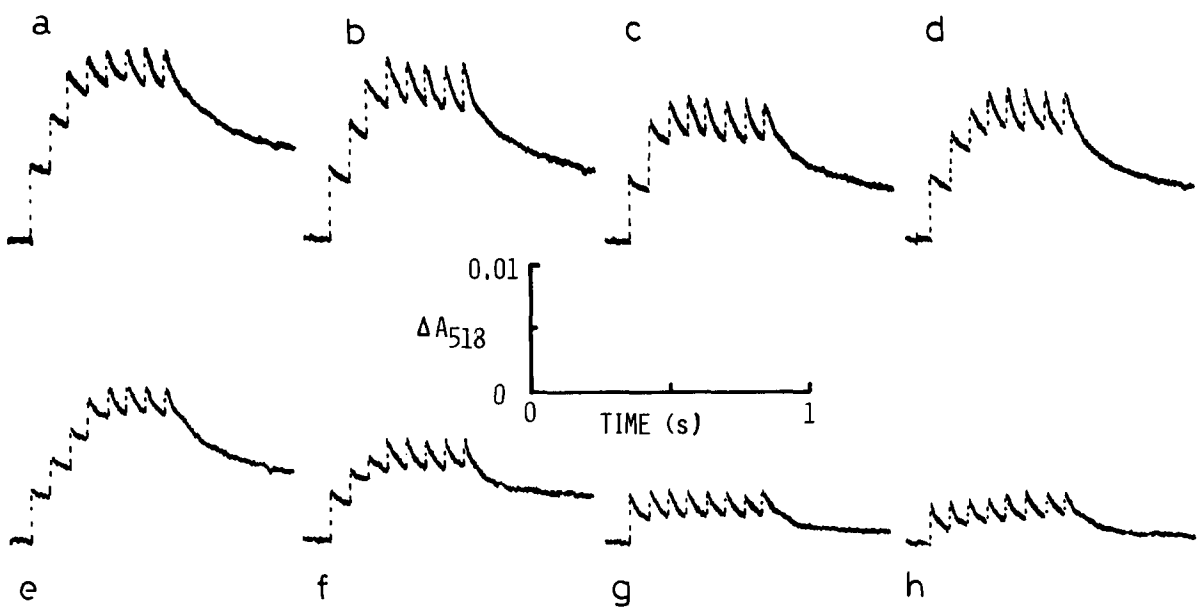

Fig. 1. Effects of ADP and ATP on the kinetics of ΔA_{518} in dark (traces a–d) and light chloroplasts (traces e–h). 0.2 mM ADP (b and f) or 0.025 mM ATP (d and h) was added in the dark 3 min before the measurements. In c and g, samples were preilluminated with 40 flashes (15 Hz) in the presence of 0.2 mM ADP at 5 min before the measurement . In a and e, there were no additions. The reaction mixture contained 50 mM KCl, 3 mM phosphate and 5 mM $MgCl_2$ and was adjusted to pH 8.0. Concentrations of dark and light chloroplasts were equivalent to 30 and 26 μg chlorophyll/ml, respectively.

ter (Union Giken RA-1201) at 17°C as described previously [6,9].

ATP hydrolysis activity was determined by measuring P_i after incubating the chloroplasts with ATP in the presence of NH_4Cl as previously described [6].

Chlorophylls were measured according to the method of Arnon [10].

Results

Chloroplasts rapidly isolated from preilluminated leaves (light chloroplasts) show more than 20-times larger ATP hydrolysis activity than those isolated from dark-adapted leaves (dark chloroplasts) [6,7,9]. Typical ATP hydrolysis activities of 8 and 216 μmol/mg chlorophyll per h were obtained for dark and light chloroplasts, respectively, in the present study. The high activity of light chloroplasts was maintained for more than 1 h as long as the chloroplasts were stored on ice.

ΔA_{518} induced by successive flashes is shown in Fig. 1. Very slow decays were observed in both dark and light chloroplasts, indicating low passive ion permeability of the membranes (Fig. 1, traces a and e). In both dark and light chloroplasts, addition of ADP in the presence of Mg^{2+} and P_i in the dark slightly changes the kinetics (traces b and f). Prolonged dark incubation (20 min) with ADP only slightly affected the kinetics (not shown). When the measurements were done after preilluminating flashes in the presence of ADP, the steady-state extents of ΔA_{518} were lowered significantly due to the acceleration of the dark decay rate. This accelerating effect was almost saturated after preillumination with 40 flashes and was observed for more than several minutes after the illumination (traces c and g). Concentrations of ATP produced after the 40 flash excitations were estimated to be 0.80 and 0.67 μM in the reaction mixtures of light and dark chloroplasts, respectively (Morita, S. and Itoh, S., unpublished data). Addition of ATP, on the other hand, accelerated the decay even when added in the dark and depressed the steady-state extent of ΔA_{518} (traces d and h). In light chloroplasts, the effects of ADP after preillumination and of ATP were more marked than those in dark chloroplasts. This tendency was also clearly seen after the single-flash excitations (Fig. 2).

The decay kinetics after one or three flash excitations were further examined in Fig. 3 by

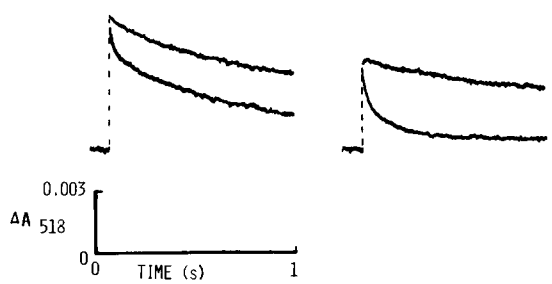

Fig. 2. Effects of ADP on the decay kinetics of ΔA_{518} after a single flash excitation. Conditions for the measurements were similar to those in Fig. 1. Left, dark chloroplasts; right, light chloroplasts. Upper trace, no additions; lower trace, +ADP (samples were preilluminated with 40 flashes).

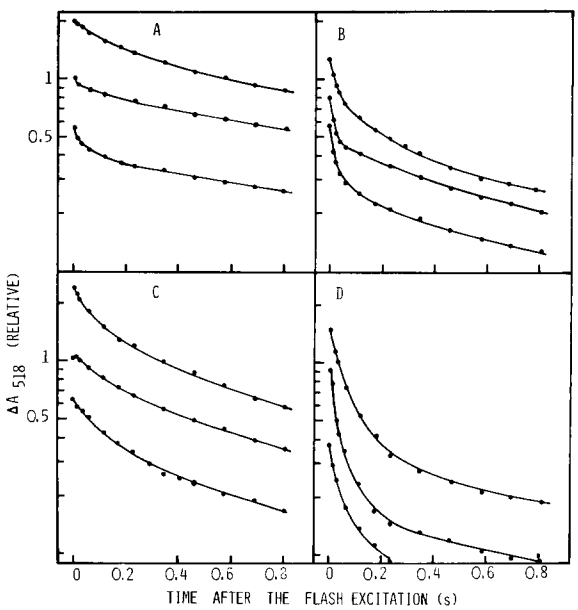

Fig. 3. Semilogarithmic plots of the decay kinetics of ΔA_{518}. Decay kinetics after three successive flashes of 75 Hz (upper trances in each figure), after one flash (middle traces) and after one flash of 30% intensity (lower traces) were measured in dark and light chloroplasts and plotted semilogarithmically against dark time after the last flash excitation. In B and D, chloroplasts were preilluminated with 40 flashes (15 Hz) in the presence of 0.2 mM ADP to achieve the maximum effects of ADP and then kept in the dark for more than 5 min before the measurements. In A and C, there were no additions. Other conditions were similar to those in Fig. 1. (A, B) Dark chloroplasts, (C, D) light chloroplasts.

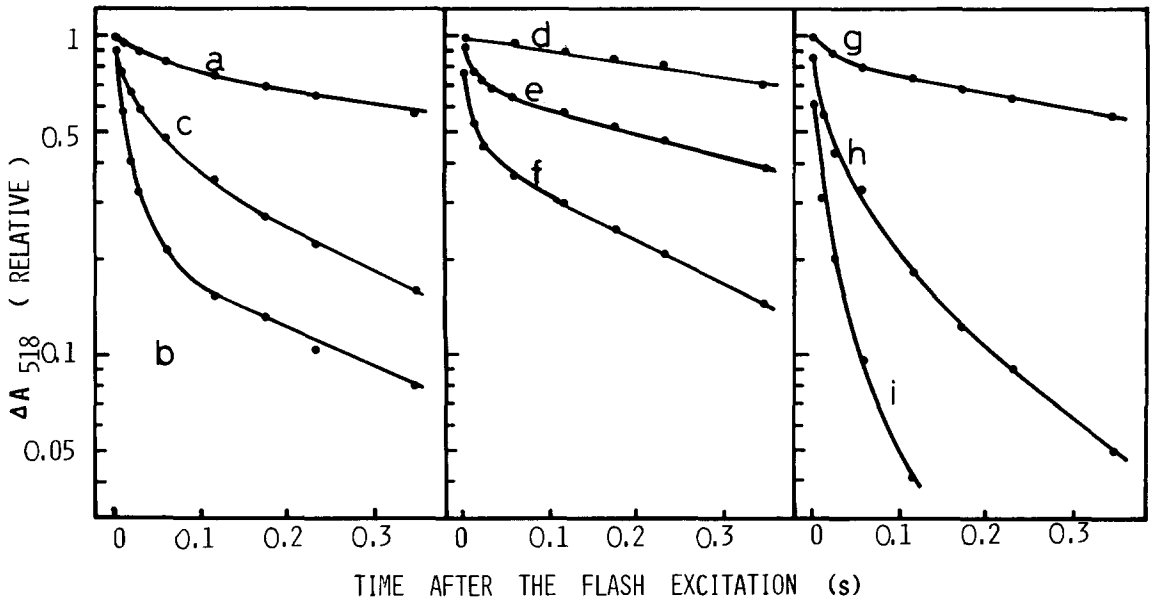

Fig. 4. Effects of DCCD, NaSCN and valinomycin on the decay kinetics of single flash-induced ΔA_{518} in light chloroplasts. (a) No addition. (b) a + 0.1 mM ADP after preillumination with 40 flashes. (c) b + 0.2 mM DCCD. (d, e and f) 0, 3 and 5 mM NaSCN added, respectively. (g, h and i) 0, 0.05 and 0.1 μM valinomycin added, respectively. Other conditions were similar to those in Fig. 1.

plotting the decay curves semilogarithmically against dark time after the last flash. Without ADP, the decay kinetics mainly consisted of a decay phase with a half-decay time of 300–500 ms in both dark and light chloroplasts. The half-time varied a little from preparation to preparation and became shorter with increase in time after chloroplast isolation. Incomplete washing of chloroplasts after the disruption of leaf segments also resulted in faster decay. The decay rate did not depend on the initial amplitide of ΔA_{518} (Fig. 3A and C). In the presence of ADP after preillumination, the rapid decay phase with a half-decay time of 15 ms became apparent and dominant in both types of chloroplasts. The decays became biphasic. The

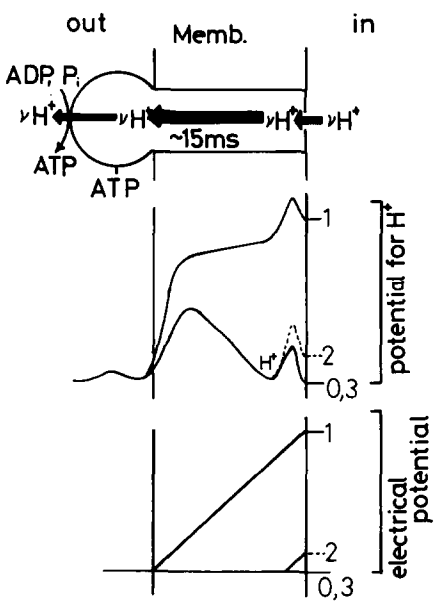

Fig. 5. Schematic model for proton movement through the CF_1-CF_0 ATP synthetase. Upper; schematic model for proton movement inside ATP synthetase. The thickness of the arrows represents the relative rate of proton movement within the membrane for each step. Middle; change of potential energy for protons. 0, 1, 2 and 3 represent the states before the flash excitation, just after the flash excitation, after the field-induced movement of protons inside the membrane which is completed within a few tens of milliseconds, and several seconds after the flash excitation when proton concentrations inside potential wells are in an equilibrium with those in the inner and outer aqueous medium, respectively. Lower; change of electrical potential across the membrane. Symbols 0 to 3 indicate the respective states as above. Contributions of surface potential due to the fixed negative charges on both sides of the membrane can be assumed to be low in the presence of Mg^{2+} and were neglected in the figure.

relative extent of the rapid phase was much larger in light than in dark chloroplasts (Fig. 3B and D). The ratio of the amplitude of the rapid phase to that of the slow phase was slightly affected by the changes in the initial extent of ΔA_{518} in each type of chloroplasts.

This ADP-induced rapid decay (preillumination without ADP did not induce rapid decay phase) seems to be related to ATP synthesis, since this phase was only seen under phosphorylating conditions or in the presence of ATP (hydrolysis of added ATP also will induce phosphorylating conditions). Treatment of chloroplasts with DCCD eliminated this rapid phase in both dark (not shown) and light chloroplasts (Fig. 4 trace c), confirming the above idea.

Addition of the membrane-permeant anion SCN^- also induced a clear biphasic decay (Fig. 4, traces e and f): the initial rate of the decay was significantly increased but not that of the slow decay phase. Addition of valinomycin (in the presence of K^+) accelerated the decay, confirming that the absobance change reflects the membrane potential change (Fig. 4, traces h and i). However, the accelerated decay did not show a biphasic nature. The difference between the actions of the valinomycin-K^+ complex and of SCN^- may give a clue as to the mechanism of proton conduction through ATP synthetase.

Discussion

Decay of membrane potential and ATP hydrolysis activity

In the ordinary class II chloroplasts (probably almost equivalent to dark chloroplasts in the present study), phosphorylating conditions are known to accelerate the field-indicating ΔA_{518} decay [3–5,11,12]. In the present study the acceleration of ΔA_{518} decay under phosphorylating conditions was more marked in light chloroplasts than in dark chloroplasts. Thus, the acceleration seems to depend on the amount of ATP synthetase with active ATP hydrolysis activity on the membrane. It may be concluded that the proton-efflux rate coupled to phosphorylation is faster in ATP synthetase with high ATP hydrolysis activity. It may be further concluded that only ATP synthetase with high ATP hydrolysis activity can rapidly undergo phosphorylation and accelerate the field decay by proton efflux.

Although dark chloroplasts carry out ATP synthesis at a high rate under continuous illuminations, or under repetitive flash excitation, the amount of active ATP synthetase will depend on the extent of $\Delta\tilde{\mu}_{H^+}$ formed by light as studied by Gräber and Witt [5] and is assumed to be rather small after a single or a few excitation flashes as used in this study. On the other hand, most of the ATP synthetase in light chloroplasts is assumed to be activated almost permanently through the action of the thioredoxin system, as recently studied by Mills and Mitchell [8] by using intact chloroplasts. This situation in light chloroplasts will result in a higher proton-efflux rate through ATP synthetase coupled to phosphorylation in a DCCD-sensitive manner and in a faster decay of the membrane potential. Light chloroplasts actually showed about 30% higher phosphorylation activity when turnover of ATP synthetase was rate limiting, i.e., under strong illumination or repetitive flash excitations (Morita, S., Itoh, S. and Nishimura, M., unpublished data).

The results in the present study suggest that the activation of ATP hydrolysis activity is directly related to the activation of ATP synthesis activity of ATP synthetase as originally suggested by Mitchell [1].

ATP requirement of the activation of ATP synthetase

The effect of ADP in acceleration of ΔA_{518} required preillumination. One interpretation for this requirement may be that the acceleration requires a large light-induced $\Delta\tilde{\mu}_{H^+}$. However, $\Delta\tilde{\mu}_{H^+}$ is expected to decrease rapidly after the end of illumination and is assumed to be very low at the time of measurement. Actually, almost negligible extents of ΔpH were assumed to exist in the dark longer than 2 min after the preillumination flashes both in dark and light chloroplasts, from measurement of the quenching of 9-aminoacridine fluorescence under similar experimental conditions (Morita, S. and Itoh, S., unpublished data). The low amount of ATP formed by the preilluminating flashes did not seem to be sufficient to maintain a large $\Delta\tilde{\mu}_{H^+}$ in the dark in the presence of excess ADP. The requirement for illumination may be

interpreted by the known characteristics of ATPase that the binding of ATP to the regulatory site is required for activation [14]. That the small amount of ATP increased the decay without preillumination also seems to evidence this interpretation. However, further work is required to determine whether the acceleration of field decay observed requires ATP binding to the noncatalytic binding site or to the catalytic site itself.

Biphasic field decay and model for proton movement in ATP synthetase

Phosphorylating conditions induced biphasic decay of ΔA_{518} with rapid (15 ms half-decay time) and slow (300–500 ms half-decay time) phases. The rapid phase is different from that induced by the breakdown of membranes, which became apparent as the aging of chloroplasts and showed a half-decay time of about 1 ms. The ratio of the rapid to the slow phase was almost independent of the initial amplitude of ΔA_{518} but was dependent on the amount of active ATPase. This result suggests that the 15 ms decay phase corresponds to proton movement inside the membrane coupled to ATP synthesis. The result provides strong evidence for the mechanism that ATP synthetase has a fixed turnover time as proposed by Witt and co-workers [12]. They estimated that the active fraction of the enzyme changes depending on the externally applied field strengths [12]. In the present study, the active fraction of ATP synthetase was directly changed by prior in vivo activation and the same conclusion was reached.

Similar biphasic decay was also induced by SCN^- but not by the valinomycin-K^+ complex. According to recent studies [15,16], membrane-permeant anions seem to meet different potential barriers from those of cations when they permeate across the membrane, having potential minimum wells just beneath the surfaces on both sides of lipid bilayer [15]. In a rough interpretation, application of the electrical field, at first, induces the rapid movement of anions from one well to the other across the hydrophobic moiety of the membrane, and then the population of anions in each well slowly (due to the charge interactions) equilibrates with the outer medium on either side of the membrane [15]. Thus, the rapid decay of the electric field seen in the presence of SCN^- in this study can be explained as reflecting the rapid decay of the electric field due to the movement of SCN^- between the wells inside the membrane.

The biphasic ΔA_{518} decay seen under phosphorylating conditions may be explained in a similar way by postulating potential wells for protons inside the CF_0 moiety of ATP synthetase just beneath the membrane surface (upper drawing in Fig. 5). The well close to the inner surface is separated from the inner aqueous medium by a diffusion barrier. The potential well on the outer CF_1 side of the membrane may be less significant. Upon the application of a membrane potential, rapid movement of protons from the inner to the outer well in the proton channel inside the CF_0 moiety is expected to occur (transition from state 1 to state 2 in middle and bottom drawings in Fig. 5). This will induce a rapid but partial dissipation of the light-induced electric field. The conformation change of the CF_1 moiety, coupled to proton movement, will liberate ATP into the aqueous medium subsequently, and then disequilibration of proton concentrations in the wells will disappear slowly due to slow equilibration with the outer aqueous media, thereby dissipating the residual electric field, which is inactive in moving protons between the wells in other ATP synthetase units but affects the equilibrium of protons between each well and the outer aqueous medium. The pH change of the outer medium will change the proton concentrations within the wells through slow equilibration and will affect the number of protons translocatable within the membrane. Inactive ATP synthetase may have a larger energy barrier, which can only be surpassed with the aid of a $\Delta\tilde{\mu}_{H^+}$ larger than a certain threshold value, between the wells. Depletion of the CF_1 moiety, on the other hand, will result in the loss of potential wells as well as the energy barrier and will induce faster proton release [13]. The proton well in Fig. 5 may be replaced by the potential well for OH^-.

This model requires no special direct interaction between the electron-transport chain and ATP synthetase and still seems to explain the biphasic decay of the intramembrane electric field under phosphorylating conditions and its dependence on the amount of active ATP synthetase seen in the present study. An increase in the amount of active ATP synthetase will result in an increase in the

number of potential wells, i.e., increase in the concentration of protons which can be immediately translocatable after the flash across the membrane. Such a situation will increase the rapid decay phase of ΔA_{518} coupled to ATP synthesis with little change in the decay rate constant (i.e., with little change in the rate of proton movements between the wells in each ATP synthetase) according to the model. The model also gives an interpretation for the inactivity of the slow decay component of the intramembrane electric field in ATP synthesis seen in bacterial chromatophores [16]. Although the situation in a small vesicle like a bacterial chromatophore may be a little different from that in the large thylakoid vesicle, and may induce localized direct interaction between electron-transport components and ATP synthetase [17], an attempt to change the active fraction of ATP synthetase in such system may give new insights into the mechanism of phosphorylation.

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