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Proton slip in the ATP synthase of *Rhodobacter capsulatus*: induction, proton conduction, and nucleotide dependence

Boris A. Feniouk[1], Armen Y. Mulkidjanian, Wolfgang Junge*

Division of Biophysics, Faculty of Biology/Chemistry, University of Osnabrück, D-49069 Osnabrück, Germany

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Abstract

F_OF_1-ATP synthase converts two energetic "currencies" of the cell (ATP and protonmotive force, *pmf*) by coupling two rotary motors/generators. Their coupling efficiency is usually very high. Uncoupled proton leakage (slip) has only been observed in chloroplast enzyme at unphysiologically low nucleotide concentration.

We investigated the properties of proton slip in chromatophores (sub-bacterial vesicles) from *Rhodobacter capsulatus* in the single-enzyme-per-vesicle mode. The membrane was energized by excitation with flashing light and the relaxation of the transmembrane voltage and pH difference was photometrically detected. We found that: (1) Proton slip occurred only at low nucleotide concentration (<1 μM) and after pre-illumination over several seconds. (2) Slip induction by *pmf* was accompanied by the release of ≈0.25 mol ADP per mole of enzyme. There was no detectable detachment of F_1 from F_O. (3) The transmembrane voltage and the pH difference were both efficient in slip induction. Once induced, slip persisted for hours, and was only partially reverted by the addition of ADP or ATP (>1 μM). (4) There was no *pmf* threshold for the proton transfer through the slipping enzyme; slip could be driven both by voltage and pH difference. (5) The conduction was ohmic and weakly pH-dependent in the range from 5.5 to 9.5. The rate constant of proton transfer under slip conditions was 185 s^{-1} at pH 8.

Proton slip probably presents the free-wheeling of the central rotary shaft, subunit γ, in an open structure of the $(\alpha\beta)_3$ hexagon with no nucleotides in the catalytic sites.

Keywords: ATP synthase; Proton slip; Regulation; *Rhodobacter capsulatus*

1. Introduction

F_OF_1-ATP synthase converts two major bioenergetic "currencies" of the cell: ATP and protonmotive force (*pmf*). *Pmf* drives proton flux through the enzyme; this flux induces conformational changes in the enzyme that are coupled to ATP synthesis. Vice versa, ATP hydrolysis can serve as energy input for proton pumping. The enzyme is located in bacterial plasma membrane and in coupling membranes of chloroplasts and mitochondria.

ATP synthase is composed of two portions: the hydrophilic F_1-portion catalyses ATP synthesis/hydrolysis and the membrane-embedded F_O-portion performs proton transfer. Both portions are multisubunit complexes. In bacteria F_1 is composed of five types of subunits in stoichiometry $\alpha_3\beta_3\gamma_1\delta_1\varepsilon_1$; F_O is a complex of $a_1b_2c_n$ subunits. The *c*-subunits form a ring-shaped oligomer; their stoichiometry in bacterial enzyme is uncertain and ranges between 8 and 11 [1,2]. F_O and F_1 portions are connected by two "stalks" that are formed by the centrally located γε and by the eccentrical b_2 dimer (for the latest structural reviews, see Refs. [3–5]).

Abbreviations: *Pmf*, protonmotive force; EDTA, Ethylene Diamine Tetraacetic Acid; BSA, bovine serum albumin; RC, photosynthetic reaction center of purple phototrophic bacteria; MES, 2-(*n*-morpholino) ethane sulfonic acid; bc_1, ubiquinol:ferricytochrome *c* oxidoreductase (cytochrome bc_1 complex); $\Delta\psi$, transmembrane voltage

* Corresponding author. Abt. Biophysik, FB Biologie/Chemie, Universität Osnabrück, D-49069, Osnabrück, Germany. Tel.: +49 541 9692872; fax: +49 541 9692262.

E-mail address: junge@uos.de (W. Junge).

[1] Permanent address: A.N. Belozersky Institute of Physico-Chemical Biology, Moscow State University, Moscow, 119899, Russia.

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The unique feature of this enzyme is the rotary catalytic mechanism: proton flux driven by *pmf* causes the rotation of the ring-shaped *c*-subunit oligomer (and of subunits γ and ε bound to it) relative to the rest of the enzyme [6–12]. In turn, the rotation of the asymmetric subunit γ inside the F_1-portion induces conformational changes that lead to ATP release and binding of ADP and phosphate (for details on the catalytic mechanism, see Ref. [13] and references therein).

The studies of the chloroplasts ATP synthase have revealed that a considerable proton transport occurs through the enzyme in the absence of nucleotides [14–17]. This transport was named "slip" to distinguish it from the "coupled" proton flow through F_OF_1, and from futile proton flow through the bare F_O-portion after F_1 has been stripped off. A protective role of the proton slip for the thylakoid membrane as an over-voltage valve has been proposed [16], and has been rejected because slip is blocked by ADP or ATP in concentration over 0.1 μM, a condition which is always met in the chloroplast stroma [17].

Our previous studies have revealed that proton slip also occurs in the enzyme from *Rhodobacter capsulatus* [18], and that it is induced by continuous illumination [19]. *Rb. capsulatus* chromatophores have been established as a very convenient system for studies of proton transport through ATP synthase. Their major advantages are: (1) stepped generation of a *pmf*-jump by excitation with a short flash of light; (2) read-out of the voltage- and ΔpH-relaxation by absorption band-shifts of intrinsic carotenoids (electrochromism) and added pH indicators; (3) single-enzyme per vesicle data analysis: owing to small size the vesicles contain on the average less than one copy of F_OF_1 [20]. This has been exploited to determine the proton conductance of F_O after stripping off F_1 [21].

In this work, we investigated the induction of slip in the F_OF_1-ATP synthase and the proton conducting properties of slipping enzyme.

2. Materials and methods

2.1. Cell growth and chromatophore preparation

Rb. capsulatus cells (wild type, strain B10) were grown photoheterotrophically on malate as the carbon source at +30 °C [22,23] as described elsewhere [19]. Chromatophores were prepared by sonication as described in Ref. [21] and washed by resuspension in 20 mM glycylglycine–KOH (pH 7.4), 5 mM $MgCl_2$, 50 mM KCl, 10% sucrose and centrifugation (180 000×*g*, 90 min, +4 °C). Special care was taken to avoid even slightest illumination of the chromatophores during preparation: all procedures were done in almost complete darkness, including the sonication step and the freezing of chromatophores in liquid nitrogen before storage. Chromatophores with F_1 stripped off from F_O were prepared by EDTA treatment as in Ref. [21]. The bacteriochlorophyll concentration in stock chromatophores was 0.5–1.5 mM. Chromatophore stock was kept at −80 °C until use.

2.2. Flash-spectrophotometric measurements

The kinetic flash-spectrophotometer was described elsewhere [21]. Saturating actinic flashes were provided by a xenon flash (full width at half maximum 10 μs) with a red optical filter (RG 780, SCHOTT, Mainz); the energy density per pulse on the cuvette was 12 mJ/cm^2. Electrochromic carotenoid band-shifts at 522 nm were used as a molecular voltmeter to monitor $\Delta\psi$ (see Ref. [21] and references therein). The calibration of electrochromic signals measured at 522 nm in millivolts of $\Delta\psi$ was done by imposing a diffusion potential of potassium cations in the presence of valinomycin as in Ref. [21].

The standard medium contained 20 mM glycine, 20 mM glycylglycine, 20 mM Na_2HPO_4, and 20 mM MES to buffer the pH in the interval from 6 to 10. Potassium chloride or acetate (100 mM) and magnesium chloride or acetate (5 mM) were present to keep the ionic strength close to the physiological value. $K_4[Fe(CN)_6]$ (2 mM) and 5 μM 1,1′-dimethylferrocene were used to maintain the redox potential of ≈+300 mV in the sample; 2 mM KCN was present to ensure that no *pmf* was generated in the darkness by cytochrome *c* oxidase. The final concentration of bacteriochlorophyll in the cuvette was 8–12 μM; residual adenine nucleotide concentration was below 20 nM. Special care was taken to avoid sample illumination during preparation. Eight transient signals at 522 nm at a repetition rate of 0.08 Hz were averaged.

Changes of the pH inside and outside the chromatophores (ΔpH_{in} and ΔpH_{out}) were monitored by pH indicators Neutral Red [24] and Cresol Red [25] as in Ref. [21]. Chromatophores were washed from the pH buffer by resuspension in 5 mM $MgCl_2$, 100 mM KCl, pH 8.0, and centrifugation (180 000×*g*, 90 min, +4 °C). The pH-buffers were omitted from the suspending medium; BSA as an impermeable pH-buffer was added to 0.3% (w/v) for the measurements of pH_{in}-transients using Neutral red [24].

2.3. Slip induction

To induce proton slip in the ATP synthase, the sample was exposed for 40 s to saturating light provided by a slide-projector with a red optical filter (OG590, Schott, Mainz) installed. Neutral density optical filters were mounted on the slide projector to attenuate the actinic light when necessary.

2.4. Measurement of nucleotide release during actinic illumination

ATP release in response to the actinic illumination was measured by the luciferin–luciferase system [26]. The medium contained 20 mM glycylglycine, 100 mM

potassium acetate, 5 mM magnesium acetate, 2 mM $K_4[Fe(CN)_6]$, 5 μM 1,1′-dimethylferrocene, 2 mM KCN, 0.2 mM luciferin and 10 U/ml luciferase; pH was 7.9. Samples were exposed for 40 s to saturating actinic illumination, and the luciferase emission was measured. Freshly prepared ATP solution was used for calibration; a linear calibration curve was obtained from 1 to 40 nM. ATP regenerating system (7.4 U/ml pyruvate kinase and 0.5 mM phospho*enol*pyruvate) was added to convert ADP to ATP and thereby determine the ADP concentration in the sample.

2.5. Other methods

F_OF_1 was purified from *Rb. capsulatus* chromatophores according to Ref. [27]. The protein concentration was determined by the Bradford assay [28]. SDS-PAGE was performed according to Ref. [29]; Coomassie staining was used for protein visualization.

3. Results

3.1. Flash-induced charge transfer reactions observed in Rb. capsulatus chromatophores

Fig. 1 shows the flash-induced electrochromic transients at a wavelength of 522 nm in chromatophores of *Rb. capsulatus* strain B10. These absorption changes reflect transients of the transmembrane electric potential difference, $\Delta\psi$, which in turn is proportional to the net charge transfer across the chromatophore membrane (see Ref. [21] and references therein).

The sharp stepwise increase observed immediately after the flash reflects the charge separation in the photosynthetic reaction centers (RCs). The charge separation in RCs is fairly independent of changes in pH, redox potential and temperature over a wide range [30]; it was used to normalize the experimental traces obtained under different experimental conditions.

When myxothiazol, a potent inhibitor of the cytochrome bc_1-complex (bc_1), was present (Fig. 1A and B), this stepwise increase in $\Delta\psi$ was the only component of the flash-induced *pmf*. Under these conditions, proton transport through ATP synthase was the only electrogenic reaction going on the millisecond time scale. This greatly simplified the interpretation of the results, in contrast to the experiments without myxothiazol (Fig. 1C and D), where the proton pumping performed by bc_1 interfered with the proton transport through ATP synthase.

Without myxothiazol, an additional slower rise of the signal (Fig. 1C and D) that reflects the electrogenic reactions of the bc_1 was observed (see Refs. [30,31] for reviews on the $\Delta\psi$ generation by the RC and the bc_1). These slower electrogenic reactions are coupled to proton transfer into the chromatophore lumen [32].

The rise of the electrochromic signal is followed by the decay that reflects the relaxation of the $\Delta\psi$. When ADP and phosphate are present in the medium, or when F_1 is stripped off from F_O, an increase in the rate of the decay is observed due to proton transport through $F_O(F_1)$ [18,21].

We used oligomycin, a potent inhibitor of F_O [33–35], to obtain the electrochromic traces corresponding to the charge transfer through $F_O(F_1)$: the difference trace±oligomycin reflects the total charge transport through F_O, irrespective of whether it was coupled to the catalytic activity in F_1 or not.

As we have previously shown, there is on average less than one active ATP synthase per vesicle in our chromatophore preparations [20,21]. It implies that the ±oligomycin difference trace was attributable to a subset of chromatophores containing one single active ATP synthase per vesicle. Therefore, the time constant of the ±oligomycin trace relaxation reflects the kinetics of proton transport through synchronized ensemble of single enzymes.

3.2. Slip induction

If special care was taken to avoid illumination of the chromatophores during preparation and storage, there was no detectable oligomycin-sensitive component of the $\Delta\psi$ decay in the absence of nucleotides, implying 100% coupling and absence of slip in the intact F_OF_1 (Fig. 1A and C). However, as we have previously noted [19], saturating pre-illumination has induced a fast, oligomycin-sensitive, component of the $\Delta\psi$ decay (Fig. 1B and D).

We have previously shown that this component is correlated with the transmembrane proton transport; its sensitivity to DCCD and venturicidin suggests that this component reflects proton transfer through the F_O-portion of the ATP synthase [18]. This component was insensitive to efrapeptin, an inhibitor that blocks F_1 [36,37], and was present in the absence of ADP and phosphate. Such a proton transfer has already been described for the chloroplast ATP synthase and is named “proton slip” [17]. We found that the slip induction required illumination for relatively long time: the half-maximal effect was observed at ≈3.5 s pre-illumination; no difference in the slip induction was observed upon illumination for longer than 40 s. We used pre-illumination for 40 s throughout the experiments.

We found that slip induction was abolished when oligomycin was added before the actinic illumination (not documented). Efrapeptin, on the contrary, has no significant effect on slip induction (Table 1). This finding is in line with the earlier observation made on the chloroplast enzyme, where slip was prevented by DCCD, but not by tentoxin—a potent inhibitor of the chloroplast F_1 activity [16].

3.3. Both Δψ and ΔpH induced slip

The *pmf* generated in chromatophores by the actinic illumination is composed of the electrical ($\Delta\psi$) and the chemical (ΔpH) component. To study the individual role of

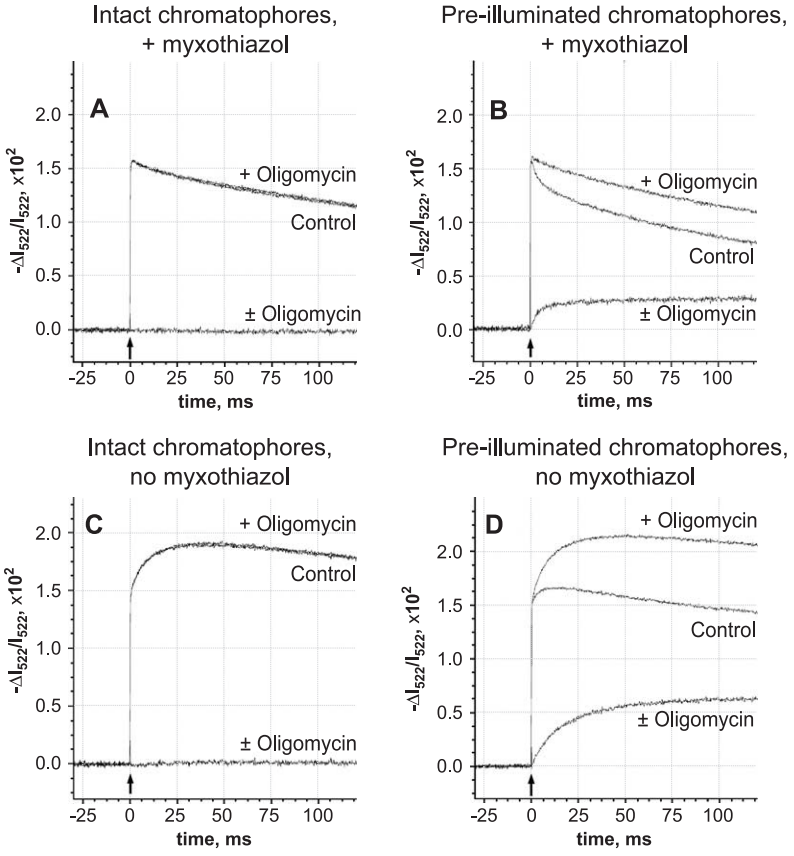

Fig. 1. Light-induced electrochromic transients in *Rb. capsulatus* chromatophores. Medium contained 100 mM KCl, 20 mM Gly, 20 mM GlyGly, 20 mM MES, 20 mM Na_2HPO_4, 5 mM $MgCl_2$, 2 mM KCN, 2 mM $K_4[Fe(CN)_6]$, 5 μM DMF; pH 8.0. Actinic flashes are indicated by arrows. The 'Control' trace in each panel was recorded immediately before oligomycin addition (3 μM), and the '+Oligomycin' trace was recorded 4 min after; '±Oligomycin' is the difference trace (see text for the details). (A) Intact chromatophores, no nucleotides, no pre-illumination, 3 μM myxothiazol present; B. Intact chromatophores were pre-illuminated for 40 s in the absence of nucleotides; then 3 μM myxothiazol was added. (C) Same as A, but no myxothiazol present. (D) Same as B, but no myxothiazol present.

$\Delta\psi$ and ΔpH in slip induction, we used the electroneutral K^+/H^+-exchanger nigericin to dissipate ΔpH and the K^+-carrier valinomycin to dissipate $\Delta\psi$. The efficiency of nigericin as a ΔpH quencher was checked by monitoring the absorption changes of amphiphilic pH-indicator Neutral Red at 545 nm [24]. Under the experimental conditions used, 1 μM nigericin quenched ≈95% of the Neutral Red response during 40 s constant actinic illumination, which was

Table 1
Slip induction

Experimental conditions	Relative extent of slip (%)
Control: Illuminated and measured at pH 8.0	100
Illuminated in the presence of 200 nM efrapeptin	86±10
Illuminated in presence of 1 μM nigericin	112±6
Illuminated in the presence of 1 mM AMP-PNP	4±4
Illuminated in the presence of 3 μM myxothiazol	37±9
Illuminated, incubated for 2 h, and measured	82±5
Illuminated at pH 8.1, incubated for 2 h with 1 mM ADP, and measured	24±2
Illuminated at pH 8.1, incubated for 2 h with 1 mM ATP, and measured	33±7
Illuminated at pH 8.1, measured at pH 6.4	38±11
Illuminated at pH 8.1, incubated at pH 6.4 for 5 min, measured at pH 8.1	113 (single measurement)
Illuminated at pH 8.1, incubated at pH 5.6 for 2 h, measured at pH 8.1	112 (single measurement)

Medium contained 100 mM KCl, 20 mM Gly, 20 mM GlyGly, 20 mM MES, 20 mM Na_2HPO_4, 5 mM $MgCl_2$, 2 mM KCN, 2 mM $K_4[Fe(CN)_6]$, 5 μM DMF. The pH was 8.0 unless indicated otherwise. Each sample was illuminated for 40 s with saturating actinic light; then 3 μM myxothiazol was added to block the bc_1. The relative extent of slip was measured as the extent of the flash-induced electrochromic ±oligomycin difference trace at 522 nm divided by the extent of the RCs response. The relative extent of slip induced in the absence of any ATP synthase inhibitors at pH 8.0 (corrected by the small background ±oligomycin difference observed without illumination in some experiments) was taken as 100%. Each value is an average of at least three experiments, unless otherwise indicated.

routinely used for slip induction (not documented). We also checked the efficiency of 1 μM valinomycin as a means to quench $\Delta\psi$, and found that it abolished ≈95% of the electrochromic transient at 522 nm during the 40-s saturating actinic illumination (not documented).

The results presented in Table 1 indicate that the illumination in the absence of nucleotides readily induced slip in the presence of 1 μM nigericin. Illumination induced slip also in the presence of valinomycin (Fig. 2). It is noteworthy that in the presence of valinomycin, it was impossible to monitor slip by the electrochromism at 522 nm, as the flash-induced $\Delta\psi$ generation was also quenched by valinomycin. In these experiments, the only driving force for proton transport was ΔpH generated by the bc_1. The results presented in Fig. 2A and C indicate that even in the absence of actinic pre-illumination, there was a detectable oligomycin-sensitive component of the transmembrane proton transfer (it was more pronounced in Fig. 2C due to higher signal-to-noise), in contrast to the electrochromic traces presented in Fig. 1A and C. This component was also observed in the electrochromic traces recorded from the same samples without valinomycin (not documented) and was probably induced during the additional wash procedure, when chromatophores were resuspended in a pH-unbuffered solution (see Materials and methods). Another possible explanation could be partial slip induction in unbuffered samples during the measurements: the flash-induced ΔpH under these conditions is much higher than in buffered samples. Despite the presence of this component, it was evident that illumination in the presence of 1 μM valinomycin induced proton slip (Fig 2B and D).

The experimental results presented in Fig. 2 also indicated that once induced, proton slip could be driven by transmembrane ΔpH without concomitant voltage.

3.4. Voltage threshold for slip induction

We investigated whether or not there was a $\Delta\psi$ threshold for the slip induction. The electrochromic carotenoid band-shift at 522 nm was calibrated in millivolts by potassium diffusion potential using valinomycin+K^+ [21]. Slip was induced in the samples containing 1 μM nigericin by illumination for 40 s with red light of variable intensity, and the concomitant voltage generation was quantitatively

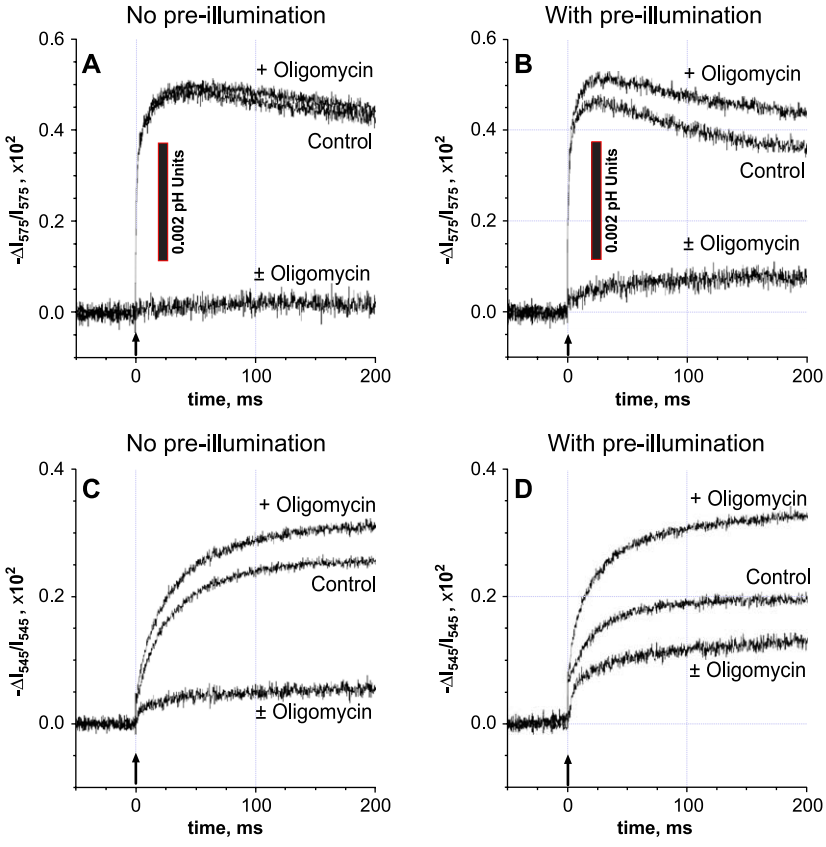

Fig. 2. Slip induction by transmembrane ΔpH. Chromatophores from the stock washed from pH-buffer were suspended in 100 mM KCl, 5 mM $MgCl_2$, 2 mM KCN, 2 mM $K_4[Fe(CN)_6]$, 5 μM DMF, 1 μM valinomycin, pH 8.0, to 10 μM bacteriochlorophyll. (A and B) pH_{out} changes. Cresol Red was added to 15 μM and flash-induced traces were measured. Corresponding control traces in the samples without the indicator were recorded and subtracted. Black bar corresponds to 0.002 pH units, according to the calibration done in the same sample. (A) No pre-illumination; (B) 40-s saturating actinic illumination before the measurements. (C and D) pH_{in} changes. 0.3% BSA was present as an impermeable pH-buffer. Neutral Red was added to 26 μM and flash-induced traces were measured. Corresponding control traces in the samples without the indicator were recorded and subtracted. (C) No pre-illumination; (D) 40-s saturating actinic illumination before the measurements.

monitored by electrochromism at 522 nm. Then the flash-induced electrochromic traces were recorded to determine the relative extent of slip. Fig. 3 shows the dependence of the slip relative extent on the voltage generated during the induction phase. The dependence was rather smooth; fit of the data with a Hill function (Fig. 4, solid line) gave a value of 150±10 mV for the half-maximum of slip induction.

3.5. Effects of nucleotide on slip induction

Fig. 4 illustrates the dependence of slip induction on the concentration of ADP and ATP. Both ADP and ATP prevented the slip induction even at concentrations lower than those necessary for full catalytic activity. We fitted the experimental data with a Hill function and obtained the values for apparent dissociation constants of 5.0×10^{-6} M for ATP and 5.0×10^{-7} M for ADP. The Hill coefficient was close to unity for both nucleotides, namely 0.8±0.2 for ADP and 1.2±0.2 for ATP.

To ensure that the observed effect of one nucleotide was not caused by traces of the other one, hexokinase+glucose was present in the experiments with ADP, and pyruvate kinase plus phospho*enol*pyruvate was present during the measurements with ATP (see caption to Fig. 4). It should be noted that in case of high concentrations of ADP, it was impossible to completely exclude the possible effects of ATP, for ATP synthesis took place during the illumination. However, as ADP prevented slip induction already at 1 μM concentration (Fig. 4), i.e., under conditions when the ATP synthesis was negligibly small, the effect observed can be attributed to ADP alone.

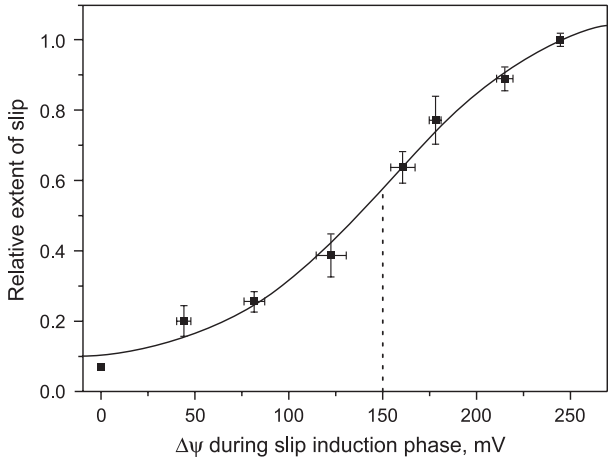

Fig. 3. Voltage threshold for slip induction. Chromatophores were suspended in 100 mM KCl, 20 mM GlyGly, 20 mM Na_2HPO_4, 5 mM magnesium acetate, 2 mM KCN, 2 mM $K_4[Fe(CN)_6]$, 5 μM DMF, 1 μM nigericin, pH 8.0. Neutral density optical filters were used to reduce the power of the actinic illumination; for each filter the transmembrane voltage generated during the illumination was measured in the presence of 3 μM oligomycin (plotted on abscissa, each point is an average of three measurements). After 40-s pre-illumination, myxothiazol was added to each sample to 3 μM, and the relative extent of the slip was measured at 522 nm as ±oligomycin difference after a single actinic flash. Solid line is the fit of the data with a Hill function.

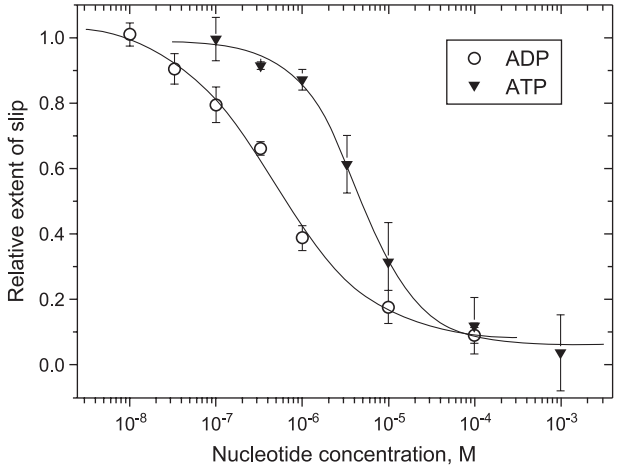

Fig. 4. Effect of nucleotides on the slip induction. Open circles—ADP; closed triangles—ATP. Nucleotides were added to chromatophores suspended in 100 mM KCl, 5 mM magnesium acetate, 20 mM glucose, 20 mM glycylglycine, 20 mM Na_2HPO_4, 2 mM KCN, 2 mM $K_4[Fe(CN)_6]$, 5 μM DMF, pH 8.0, and pre-illuminated for 40 s; myxothiazol (3 μM) and efrapeptin (250 nM) were added immediately after pre-illumination. Hexokinase (5 units/ml) was present in the experiments with ADP to avoid contamination with ATP; 7.4 units/ml pyruvate kinase and 0.5 mM phospho*enol*pyruvate were present in the experiments with ATP to prevent contamination with ADP. The extent of slip induced without the corresponding nucleotide (corrected for small ±oligomycin difference that was sometimes observed in control samples that were not illuminated) was taken as unity. Solid lines correspond to Hill function fits of the data (see text for details). Each point is an average of at least three experiments; standard errors are given as bars.

The slip induction was also efficiently prevented by AMP-PNP, a non-hydrolysable ATP analogue (Table 1).

3.6. Nucleotide release during slip induction

We investigated if the slip induction by *pmf* was coupled to the release of nucleotides. The data presented in Table 2 indicated that illumination of chromatophores led to a modest ATP release (middle bar; ≈0.025 nM ATP/μM BChl). In the presence of an ATP regenerating system, the observed ATP release increased approximately 10-fold. The result implied that illumination caused the release of ≈0.25 nmol adenine nucleotides per micromole of BChl with 90% of ADP and 10% of ATP. No ATP release upon illumination was observed in the presence of oligomycin (Table 2), indicating that the effect observed was due to the proton transfer through the ATP synthase.

Taking the figure of ≈1 mol ATP synthase per 1000 mol bacteriochlorophyll [20,38], we estimate a release stoichiometry of ≈0.25 nucleotides released per ATP synthase.

3.7. No detachment of F_1 was observed after slip induction

We addressed the question whether the slip observed after actinic illumination of chromatophores was a consequence of F_1 detachment from F_O.

Table 2
Release of nucleotides upon illumination of chromatophores

Experimental conditions	Release of ATP (nM/μM BChl)
No additions	0.025±0.007
0.5 mM phospho*enol*pyruvate	0.249±0.048
0.5 mM phospho*enol*pyruvate and 3 μM oligomycin	−0.028±0.026

Chromatophores were suspended in 100 mM potassium acetate, 5 mM magnesium acetate, 20 mM glucose, 20 mM glycylglycine, 20 mM Na_2HPO_4, 2 mM KCN, 2 mM $K_4[Fe(CN)_6]$, 5 μM DMF, 7.4 units/ml pyruvate kinase, 10 units/ml luciferase and 0.2 mM luciferin; pH was 8.0. ATP release in response to 40-s saturating actinic illumination was measured by luciferase emission (see Materials and methods).

One sample was illuminated in the absence of nucleotides, and the other one was kept in the dark and served as a control. Both samples were centrifuged at 180 000×*g* for 1 h immediately after illumination.

From the estimate of one ATP synthase per 1000 bacteriochlorophylls [20,38], it follows that if the F_1-portion was completely and irreversibly released, its concentration in the supernatant would rise to 7.1 nM, mounting the protein concentration by ≈3 μg ml^{-1}.

We also precipitated total protein from each supernatant with 15% TCA and analysed the samples by SDS-PAGE. Purified *Rb. capsulatus* F_OF_1 diluted in the same medium to the final concentration of 7 nM and precipitated with 15% TCA served as a control. As can be seen in Fig. 5 (lane B), subunits α, β and γ were clearly visible in the F_OF_1 control lane, while no traces of protein were observed in these positions in both supernatants. The results indicated that no detectable detachment of F_1 from F_O was caused during pre-illumination. It should be noted that the results obtained rule out the detachment of the whole F_1-portion, while dissociation of small subunits alone (e.g., subunit ε or δ) could well have taken place and remained undetected.

3.8. Properties of the proton slip and its pH dependence

To study the kinetic properties of the light-induced slip, we routinely inhibited the bc_1 by myxothiazol (see Fig. 1 to compare the original electrochromic traces with and without myxothiazol). This was done to avoid the overlapping of the electrogenic reactions in the bc_1 with proton transport through the ATP synthase. Both enzymes operate in the millisecond time scale, and it was impossible to clearly separate their electrogenic activities when monitoring the electrochromic transients reflecting the net $\Delta\psi$ changes averaged over the whole chromatophore vesicle ensemble. Moreover, there was also mutual influence between the electrogenic proton pumping activity of the bc_1 and the proton transfer through ATP synthase that made impossible clear-cut interpretations of the results (see Ref. [21] for details).

Another important reason to block the bc_1 was that no ΔpH was generated in response to a single flash [32]. Under these conditions, the only driving force for proton transport through ATP synthase was $\Delta\psi$, which could be directly quantitatively measured by the electrochromic transients.

A side effect of myxothiazol was a marked decrease in induction of slip by pre-illumination (see Table 1). To avoid this, the chromatophores were first pre-illuminated and myxothiazol was only added thereafter.

Once induced, the proton slip persisted. Mild decrease in the extent of slip was observed upon 2-h incubation (Table 1). This implied that under the experimental conditions used (no nucleotides), *pmf* induced a stable change in the enzyme that led to slip. When ADP and ATP were added after the slip induction, a decrease in the slip extent was observed. However, ≈25% of the initial slip was not blocked in 2 h, suggesting that the "slipping" conformation might be only partially reversible.

Fig. 6 illustrates the pH dependence of the slip. The chromatophore suspension was illuminated at pH 8 to induce slip; after that, the pH was adjusted to the desired

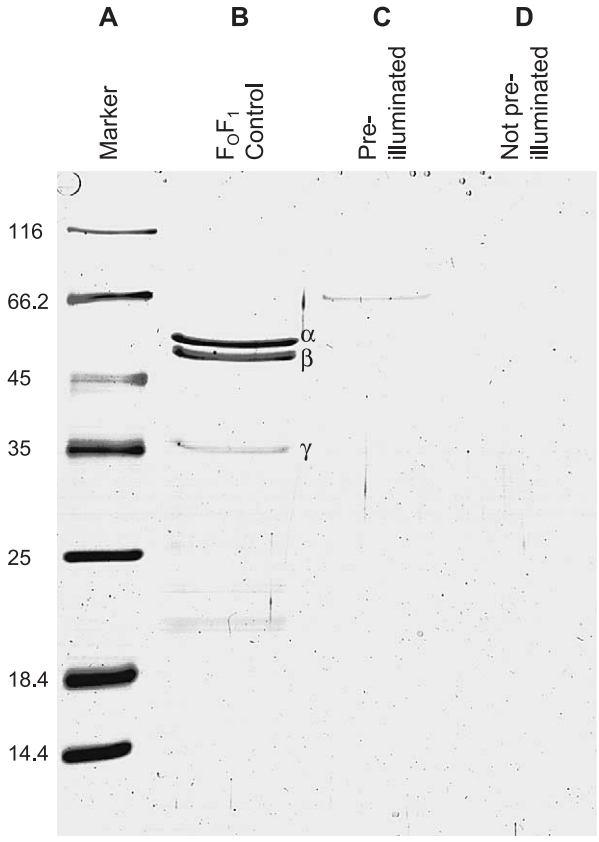

Fig. 5. Protein composition of the non-membrane-bound fraction of chromatophore suspension before and after the 40-s pre-illumination in the absence of nucleotides. Chromatophores were suspended to 7.1 μM bacteriochlorophyll in 100 mM KCl, 20 mM glycylglycine, 20 mM Na_2HPO_4, 5 mM $MgCl_2$, 2 mM KCN, 2 mM $K_4[Fe(CN)_6]$, 5 μM DMF, pH 8.0. One sample was illuminated for 40 s (lane C), the other was not and served as a control (Lane D). Samples were centrifuged; 1 ml of the supernatant was precipitated with 15% TCA and analysed by SDS-PAGE (Coomassie staining). As another control, purified *Rb. capsulatus* F_OF_1 was dissolved in the same medium to 7 nM final concentration, and 1 ml was precipitated with 15% TCA (lane B). Lane A was a protein molecular weight marker.

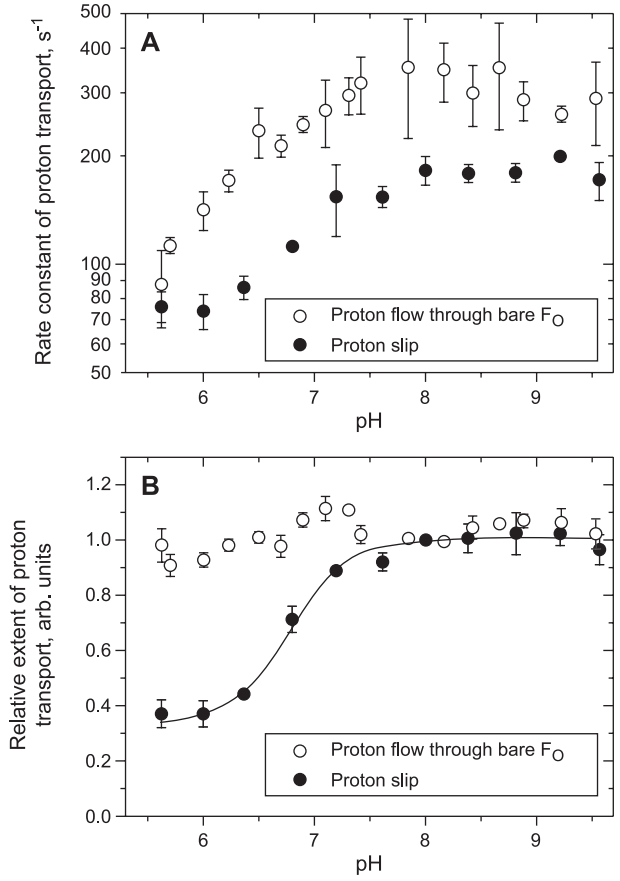

Fig. 6. Comparison of slip with proton flow through bare Fo. (A) pH dependence of the rate constants of proton slip (solid circles) and of proton transport through bare F_O (hollow circles). The constants were determined from the exponential fits of ±oligomycin difference traces measured at given pH. Data for proton transport through bare F_O stripped of F_1 were taken from Ref. [21]. (B) pH dependence of the relative extent of proton slip and of proton transport through bare F_O. Each point corresponds to the maximal extent of ±oligomycin trace at given pH normalized by the flash-induced voltage step generated by the RCs. Solid line is the fit of the data with a Hill function. Each point represents an average of at least three independent measurements; standard errors are given as bars.

value, and the measurements were done (see caption in Fig. 6 for the details).

The rate constant of the oligomycin-sensitive voltage decay under slip was almost pH-independent in the interval from 5.5 to 9.5; its value reached ≈185 s^{-1} at pH above 7.5 and gradually dropped to ≈75 s^{-1} upon acidification (Fig. 6A, solid). This pattern resembled the pH dependence of the rate constant of proton flow through bare F_O after stripping off the F_1 (Fig. 6A, open circles, taken from Ref. [21]), although the absolute value of the slip rate constant was approximately twofold lower.

The pH dependence of the relative extent of slip was markedly different from that of the proton flow through bare F_O (Fig. 6B). A drop by over 60% was observed upon acidification from pH 9.5 to 5.5, in contrast to the pH-independent extent of proton transport through F_O stripped of F_1 by EDTA treatment. We fitted the experimental points with a Hill function and obtained the apparent pK=6.8±0.1 with n=1.6±0.5. The inhibition of slip at acidic pH was reversible: when samples illuminated at pH 8 were incubated at pH 5.5 and then titrated back to pH 8, no reduction in the relative extent of slip was observed (Table 1).

3.9. Absence of voltage threshold for slip

To investigate if the rate or relative extent of the light-induced uncoupled proton flow was dependent on the absolute value of the $\Delta\psi$, we used neutral density filters to attenuate the actinic flash. Samples with low bacteriochlorophyll concentration (5 μM) were used in these experiments to avoid uneven excitation at sub-saturating flash energy. As can be seen in Fig. 7A, the voltage jump generated after a single flash dropped more then sixfold upon flash attenuation. When the traces were scaled to fit each other, no considerable changes were observed neither in the kinetics nor in the relative extent of the ±oligomycin traces (Fig. 7B). The result implied that although a relatively high *pmf* was necessary to induce slip, once induced, it had no detectable threshold *pmf* value. This conclusion was also supported by the results presented in Fig. 2 where proton slip was driven by ΔpH only without concomitant voltage.

4. Discussion

4.1. Slip induction

The results presented in Figs. 1 and 2 indicate that the *pmf* generated during sample illumination in the absence of nucleotides induced proton slip in the ATP synthase. As documented in Fig. 1A and C, if special care was taken to avoid sample illumination, there was no detectable proton flow through the enzyme in the absence of nucleotides. This result emphasizes that even occasional exposure to low light that occurred during standard preparations used in our previous works [18,19] led to considerable slip induction.

The data presented in Fig. 1D show that single actinic flashes were insufficient for slip induction. Under these experimental conditions (active bc_1, no nigericin), a single actinic flash generates ≈100 mV $\Delta\psi$ [21] plus some ΔpH (estimates of up to ≈0.3–0.8 pH units per single flash was reported [32,39]). However, Fig. 3 illustrates that slip was already induced to some extent at $\Delta\psi$ values lower then 100 mV. It implied that the induction itself had a relatively slow rate (at least slower than the decay of $\Delta\psi$ observed in Fig. 1C, i.e., the rate constant was below 3 s^{-1}). This corresponds well with illumination time of ≈3.5 s necessary to induce half-maximal slip.

The data presented in Table 2 indicated that in the absence of nucleotides *pmf* led to release of ADP and ATP bound to the ATP synthase; the sensitivity of this release to oligomycin implied that it was caused by proton translocation through F_O. This finding is in line with the results

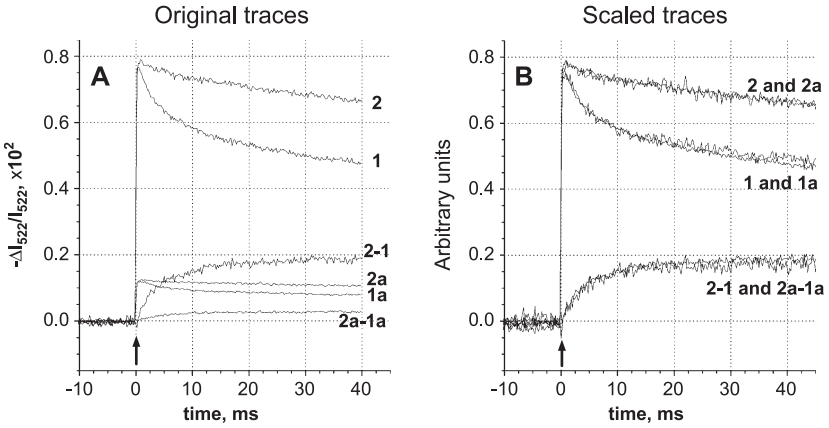

Fig. 7. Absence of voltage threshold for slip. Chromatophores suspended to 5 μM bacteriochlorophyll in 100 mM KCl, 20 mM GlyGly, 20 mM Na_2HPO_4, 5 mM magnesium acetate, 2 mM KCN, 2 mM $K_4[Fe(CN)_6]$, 5 μM DMF, 1 μM nigericin, pH 8.0, were exposed to saturating actinic illumination, then myxothiazol was added to 3 μM. (A) Same sample was excited with saturating flash (traces 1, 2 and 1–2) and attenuated flash (traces 1a, 2a and 1a–2a). Traces 1 and 1a were recorded immediately before, and traces 2 and 2a were recorded 4 min after oligomycin was added to 3 μM. Traces 1–2 and 1a–2a are difference ±oligomycin traces. (B) Difference ±oligomycin traces from panel A normalized by the extent of the fast $\Delta\psi$ jump generated by RCs (scaling factor 6.27). Actinic flashes are indicated by arrows.

obtained on the chloroplast enzyme, where up to ≈0.25–0.4 adenine nucleotides were released per ATP synthase in response to *pmf* [40–42]. The sub-stoichiometric nucleotide release might indicate that there was already a fraction of enzymes without nucleotides in the catalytic sites before the pre-illumination.

In the crystal structure determined for the F_1-portion from the thermophilic *Bacillus* PS3 with no nucleotides in the catalytic sites [43], all the β-subunits have the same "open" conformation with the DELSEED loop positioned far away from the γ-subunit, implying a weaker interaction between γ and the rest of the F_1-portion. However, such "open" conformation seems to be only a prerequisite for slip induction by *pmf*, because no detectable slip was observed before pre-illumination (Fig. 1).

Prevention of slip induction by ADP, ATP and AMP-PNP documented in Fig. 4 and Table 1 was also in line with the speculation above, because the binding of nucleotides is expected to stabilize the enzyme by keeping at least one of its β-subunits in a "closed" conformation. Such "closed" conformation was observed in all crystal structures for the β-subunits that formed a catalytic site where a nucleotide was bound [37,44–47]. The protective effect of AMP-PNP points out that nucleotide binding is already sufficient to prevent slip induction, i.e., no catalytic activity seems to be required.

Experiments on the chloroplast ATP synthase, where *pmf* was shown to weaken the F_O–F_1 interactions [48], raised a question if proton slip in *Rb. capsulatus* chromatophores was accompanied by the detachment of F_1 from F_O. Our results indicate that this was not the case. First, the rate and the pH dependence of slip extent differed from those of exposed F_O. Second, the experiments in Fig. 4 pointed out that no detectable F_1 detachment took place in response to illumination. Therefore, we find it more probable that slip took place in the whole enzyme, which became uncoupled in an "open" conformation, rather than caused by detachment of F_1 from F_O. However, our data cannot exclude partial or even complete detachment of subunit ε or δ during slip induction. In *E. coli*, enzyme subunit ε was shown to spontaneously dissociate upon dilution [49].

4.2. Slip properties

Although slip was not caused by the detachment of F_1, it resembled to some extent the proton transport through bare F_O stripped of F_1 by EDTA treatment [21]. Other common features were the absence of a voltage threshold (see Fig. 7) and the ability of the transmembrane ΔpH alone to drive proton flow (Fig. 2).

There were, however, several differences between these two modes of proton conduction. The maximal rate constant of slip was twofold lower than that of proton flow through bare F_O (see Fig. 6A). Another distinctive property was the pH dependence of the slip extent. As can be seen in Fig. 6B, at pH 6 the extent dropped by more than 60% in comparison with pH 8, while in case of the bare F_O the relative extent of proton flow was pH-independent. As there is a single molecule of ATP synthase per vesicle in those chromatophores that contain the enzyme, such drop in the extent reflected complete "switching off" of ~60% slipping enzymes, while the rest 40% were pH-insensitive. The nature of such heterogeneity remains to be solved; one possible explanation is heterogeneity of the enzyme ensemble (e.g., a partial dissociation of subunit ε took place).

The rate constant of 185 s^{-1} implied that at 100 mV voltage, the rate of proton transport through the slipping ATP synthase would reach ≈2800 or ≈5400 H^+ s^{-1} at 200 mV (compared with 6000 and 12 000 H^+ s^{-1} for exposed F_O; see Ref. [21] for the calculation details). This figure exceeds severalfold the estimated rate of proton transfer

under ATP synthesis conditions ($\approx$1000 H^+ s^{-1} [21]). The absence of the *pmf* threshold for slip and poor reversibility implied that even a single slipping ATP synthase might substantially lower the *pmf* in the whole bacterial plasma membrane. Inhibition of slip by low pH documented in Fig. 6A might provide a feedback regulatory mechanism, for proton inflow driven by *pmf* under slip conditions is expected to acidify the cytoplasm.

4.3. Comparison with the proton slip in the chloroplast ATP synthase

The earlier works on chloroplast ATP synthase revealed that there was a significant proton slip through the enzyme in the absence of nucleotides. This slip was abolished at pH below 7; ATP and ADP also blocked slip in micromolar concentrations [14,16,17]; the presence of magnesium ions was essential for this process [50]. The results presented in this work indicate that in *Rb. capsulatus* slip was also blocked at low pH. However, effects of ADP and ATP after the slip induction were different from those in chloroplasts: no immediate inhibition of slip was observed. The nucleotides prevented the slip induction by *pmf* only when present *before* pre-illumination.

The threshold *pmf* value was also not observed for the slip itself but for the induction of slip (compare results in Figs. 3 and 7). In the earlier studies, the issue of the slip induction by *pmf* was not addressed, for slip was considered to be an intrinsic property of the ATP synthase. Therefore, the nucleotide effects and $\Delta\psi$ threshold for the slip induction could not be distinguished from those for proton conduction once the slip was induced.

Confusion between slip and slip induction is the most probable explanation for the different effects of nigericin on slip in *Rb. capsulatus* (Table 1) and in chloroplast, where a marked inhibition of slip by nigericin was observed [17]. In chloroplast the ΔpH-component of the *pmf* is expected to be more important for the induction than $\Delta\psi$, which is relatively low in thylakoids. In chromatophores, on the contrary, actinic illumination leads to high $\Delta\psi$ (see Ref. [51] and Fig. 2). So even if ΔpH was quenched by nigericin, slip could be efficiently induced by $\Delta\psi$ alone.

5. Conclusions

To our knowledge, this work is the first experimental evidence of the induction of proton slip in the ATP synthase by *pmf*. This induction was slow (half-time 3.5 s), could be driven either by ΔpH or $\Delta\psi$ alone, required relatively high *pmf* (half-maximum effect at $\Delta\psi$=150 mV), and was abolished by ADP or ATP. Once induced, proton slip (i) had no detectable *pmf* threshold, and (ii) was partially blocked by ADP or ATP, as well as by low pH. It is probable that free rotation of subunit γ inside the $(\alpha\beta)_3$ hexagon in an open conformation is a prerequisite for the proton slip.

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