

# ATP-synthase of *Rhodobacter capsulatus*: coupling of proton flow through $F_0$ to reactions in $F_1$ under the ATP synthesis and slip conditions

Boris A. Feniouk<sup>a,b</sup>, Dmitry A. Cherepanov<sup>a,c</sup>, Wolfgang Junge<sup>a</sup>, Armen Y. Mulkidjanian<sup>a,b,\*</sup>

<sup>a</sup>Division of Biophysics, Faculty of Biology/Chemistry, University of Osnabrück, D-49069 Osnabrück, Germany

<sup>b</sup>A.N. Belozersky Institute of Physico-Chemical Biology, Moscow State University, Moscow, 119899, Russia

<sup>c</sup>Institute of Electrochemistry, Russian Academy of Sciences, Leninskii prosp. 31, Moscow, 117071, Russia

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**Abstract** A stepwise increasing membrane potential was generated in chromatophores of the phototrophic bacterium *Rhodobacter capsulatus* by illumination with short flashes of light. Proton transfer through ATP-synthase (measured by electrochromic carotenoid bandshift and by pH-indicators) and ATP release (measured by luminescence of luciferin-luciferase) were monitored. The ratio between the amount of protons translocated by  $F_0F_1$  and the ATP yield decreased with the flash number from an apparent value of 13 after the first flash to about 5 when averaged over three flashes. In the absence of ADP, protons slipped through  $F_0F_1$ . The proton transfer through  $F_0F_1$  after the first flash contained two kinetic components, of about 6 ms and 20 ms both under the ATP synthesis conditions and under slip. The slower component of proton transfer was substantially suppressed in the absence of ADP. We attribute our observations to the mechanism of energy storage in the ATP-synthase needed to couple the transfer of four protons with the synthesis of one molecule of ATP. Most probably, the transfer of initial protons of each tetrad creates a strain in the enzyme that slows the translocation of the following protons.

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**Key words:** Membrane potential; ATP synthesis; Elasticity; Proton transfer; (*Rhodobacter capsulatus*)

## 1. Introduction

ATP-synthase uses the transmembrane electrochemical potential difference of protons ( $\Delta\mu_{H^+}$ ) to produce ATP from ADP and  $P_i$  (see [1–3] for reviews). According to the experimental estimates, the synthesis of one ATP molecule seems to require the translocation of four protons [4–6]. ATP-synthase is composed of two domains, namely, the membrane-embedded proton-translocating  $F_0$  and the peripheral catalytic  $F_1$ . The bacterial  $F_0$  is formed by three different subunits (*a*, *b* and *c*); in *E. coli* their stoichiometry has been shown to be  $a_1:b_2:c_{12}$  with 12 *c*-subunits arranged as a ring [7,8]. The crystal structure of  $F_1$ , both in mitochondria and bacteria, shows a hexamer of  $\alpha_3\beta_3$ -subunits with a  $\gamma$ -subunit as a central shaft [9–11]. Driven by ATP hydrolysis, the  $\gamma$ -subunit

rotates inside the  $\alpha_3\beta_3$ -hexagon [12–15] in three discernible steps [16,17]. It was speculated that the  $\gamma$ - and  $\epsilon$ -subunits of  $F_1$  interact with the  $c_{12}$ -ring of  $F_0$  to form the rotor of the ATP-synthase, whereas the subunits  $ab_2$  of  $F_0$ , being connected to the  $\alpha_3\beta_3$ -hexagon by subunit  $\delta$ , form the stator [2,18,19]. It has been proposed that proton flux through  $F_0$  drives the sliding of the  $c_{12}$ -ring relative to the *a*- and *b*-subunits, and thereby the rotation of  $\epsilon$  and  $\gamma$  [2]. The rotating  $\gamma$ -subunit is assumed to open the ATP-binding sites and to cause the release of the tightly-bound ATP [9].

We investigated the coupling of proton flow to ATP synthesis in chromatophores of phototrophic bacterium *Rhodobacter capsulatus* by monitoring the proton transfer through  $F_0F_1$  and the ATP release in response to the flash-induced stepwise increase of the membrane potential. We found out that the  $H^+/ATP$  ratio decreased with the flash number from an apparent value of 13 upon the first flash to about 5 when averaged over three flashes. Possible mechanisms accounting both for this observation and for the two resolved kinetic components of proton transfer are discussed.

## 2. Materials and methods

### 2.1. Chromatophores

Chromatophores were isolated from the cells of *Rb. capsulatus* (wild-type, strain B10) grown at +30°C photoheterotrophically on malate [20]. The cells were disrupted by a Ribi-press treatment (12000 psi) [21]. The preparation medium contained: 50 mM NaCl or KCl, 10 mM  $MgCl_2$ , 10% sucrose, 0.5 mM DTT, 30 mM HEPES, pH 7.4. The measurements were conducted in the medium which contained 100 mM glycyl-glycine, pH 8.0, 50 mM KCl, 2 mM  $Na_2PO_4$ , 10 mM  $MgCl_2$  (substituted for 10 mM Mg acetate during the ATP measurements), 2 mM potassium ferrocyanide (used as a redox buffer;  $E_h$  was  $\sim 250$  mV), 10  $\mu M$  1,1'-dimethylferrocene (used as a redox mediator) and 2 mM KCN (used to prevent the oxidation of the redox-buffering system through the terminal oxidase). The concentration of bacteriochlorophyll in the samples was determined according to [22]. The amount of functionally active RCs was estimated from the extent of flash-induced absorption changes at 603 nm [23]. The amount of  $F_0F_1$  was determined by titration with venturicidin. The  $F_0F_1:RC:BChl$  ratio in our samples was about 1:6:540.

### 2.2. Spectrophotometric measurements

Spectrophotometric measurements were performed with the set-up described [24]. The time resolution was 1  $\mu s$ . Saturating exciting flashes were provided by a Xenon flash-lamp ( $\sim 4 \mu s$  FWHM, Schott RG 780 nm filter). Repetitive signals were averaged. The time interval of 8 s between flash series was sufficient for a full decay of the transmembrane voltage but short enough to keep one molecule of ubiquinol forming in about 50% of RCs after each flash (see [25]). The  $\Delta\psi$ -changes on the chromatophore membrane were monitored by electrochromic carotenoid bandshift at 524 nm [21]. pH-changes inside chromatophores were monitored by absorption changes of the amphiphilic pH-indicator Neutral red at 546 nm [21]. In these experiments 0.3% BSA was used as an impermeable buffer to quench the pH-changes in the medium. The nigericin-sensitive component of the Neutral red

\*Corresponding author. Fax: +49 (541) 9692870.

E-mail: mulkidjanian@sfbbio1.biologie.uni-osnabrueck.de

**Abbreviations:** BChl, bacteriochlorophyll; BSA, bovine serum albumin; DCCD, *N,N'*-dicyclohexylcarbodiimide; DTT, dithiothreitol; FCCP, carbonyl cyanide, *p*-trifluoromethoxyphenylhydrazone; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; RC, photosynthetic reaction center of purple phototrophic bacteria;  $P_i$ , inorganic phosphate;  $\Delta\mu_{H^+}$ , transmembrane electrochemical potential difference of protons;  $\Delta\psi$ , transmembrane electrical potential difference

absorption changes was attributed to the changes of the  $\Delta\text{pH}_{\text{in}}$ . pH-changes in the outer medium ( $\Delta\text{pH}_{\text{out}}$ ) were monitored by absorption changes of the hydrophilic pH-indicator Cresol red at 575 nm; chromatophores washed from pH-buffers were used [24]. These pH-transients were calibrated by the addition of 0.1 M KOH solution aliquots. The optical path was 2 cm, both for the exciting and the measuring beam. Final bacteriochlorophyll concentration in the cuvette was about 10  $\mu\text{M}$ .

### 2.3. ATP release

ATP release was monitored by on-line measurement with the luciferin-luciferase system as in [26]. ATP was added to concentration of 0.2–1  $\mu\text{M}$  for calibration. The photomultiplier was shielded against actinic light by a stack of three BG-39 filters (Schott, Mainz). The optical path was 1 cm. Bacteriochlorophyll concentration during these measurements was about 70  $\mu\text{M}$ .

## 3. Results

The illumination of chromatophores by a series of short flashes of light caused a stepwise increase in the membrane potential, the electrical component of which,  $\Delta\psi$ , was monitored by electrochromic carotenoid bandshift at 524 nm (Fig. 1). After each flash, two components of the increase were observed. The fast, here unresolved, increase in  $\Delta\psi$  was caused by the turnover of the photosynthetic reaction center (RC) whereas the slow component of the increase reflected the operation of the cytochrome-*bc*<sub>1</sub> complex ( $\tau \sim 10$  ms). The increase was followed by a decay reflecting the discharge of the membrane. To identify the components of the discharge which were due to the proton efflux through ATP-synthase we applied specific inhibitors of  $F_0F_1$  [27,28]. The action of inhibitors differed depending on whether ADP was present in the medium (conditions of ATP synthesis) or not.

### 3.1. Charge flow through $F_0F_1$ in the absence of ADP

(Fig. 1, top row)

The addition of specific inhibitors of  $F_0$ , namely of oligomycin, DCCD or venturicidin (see [29] for their survey) increased the magnitude of the flash-induced  $\Delta\psi$ -changes. For venturicidin it is documented in Fig. 1A. We attributed the increase to the blockage of the proton efflux through  $F_0F_1$  as it has been previously done for chromatophores of *Rb. sphaeroides* [27] and for thylakoids [28]. Fig. 1B shows that efrapentin, a peptide inhibitor of  $F_1$  which binds between  $\alpha_3\beta_3$ -hexagon and  $\gamma$ -subunit [30], was almost without effect under these conditions (trace 3). The kinetics measured in the absence of ADP were independent on the concentration of  $P_i$  (up to 5 mM, not documented).

### 3.2. Charge flow through $F_0F_1$ in the presence of ADP and $P_i$

(Fig. 1, middle row)

The extent of proton efflux was larger in the presence of ADP and  $P_i$  (synthesis conditions) than under conditions when no ADP was present (compare trace 4 in Fig. 1C with trace 1 in Fig. 1A). The half-activation by ADP was observed already at 3  $\mu\text{M}$  ADP (the respective concentration dependence is plotted in Fig. 3B, see open symbols). Under the synthesis conditions the proton flux was inhibited both by venturicidin (Fig. 1C, trace 5) and by efrapentin (Fig. 1D, trace 6). In the presence of the latter, the residual proton efflux through  $F_0F_1$  was comparable to those observed under the conditions when no ADP was present (compare trace 6 in Fig. 1D with traces 1 and 3 in Fig. 1B). Noteworthy is that the

kinetics of  $\Delta\psi$ -changes measured in the presence of venturicidin and efrapentin were independent on whether ADP was present or not (trace 2 in Fig. 1A almost coincided with trace 5 in Fig. 1C, as well as trace 3 in Fig. 1B was similar to trace 6 in Fig. 1D).

As apparent from Fig. 1A–D, the faster, venturicidin-sensitive component of  $\Delta\psi$  decay attributed to  $F_0F_1$  always disappeared at a certain level of the transmembrane voltage. The remaining slow component of the decay was unrelated to ATP-synthase. The phenomenon has been interpreted before for chloroplasts as an indication of a threshold  $\Delta\psi$  for the proton conduction via ATP-synthase [31]. According to Fig. 1, the threshold value was lower in the presence of ADP than in its absence.

### 3.3. The kinetics of charge transfer through $F_0F_1$

The kinetics of charge transfer through  $F_0F_1$  (Fig. 1, bottom) was revealed from the difference traces  $\pm$  venturicidin. Fig. 1E shows that at gross inspection the charge transfer patterns were similar with (trace 5-4) and without ADP (trace 2-1). In both cases, the stepwise increase of the transmembrane voltage caused the steps of charge efflux through  $F_0F_1$ ; the extent of efflux was smaller in the absence of ADP. A close look at the kinetic traces after the first flash at an expanded time scale (Fig. 1F) revealed a biphasic rise of the charge transfer both with and without ADP. It could be fitted by two exponential functions with time constants of  $\sim 6$  ms and of  $\sim 20$  ms, respectively. The absence of ADP suppressed selectively only the slow component.

### 3.4. Measurements of pH-changes by pH-indicating dyes

(Fig. 2)

The flash-induced changes of pH both inside and outside of chromatophores ( $\Delta\text{pH}_{\text{in}}$  and  $\Delta\text{pH}_{\text{out}}$ , respectively) were measured in the absence of ADP,  $P_i$  and pH-buffers. To discriminate the components caused by the proton transfer via  $F_0F_1$ , we took the difference between  $\Delta\text{pH}$ -changes measured in the presence of venturicidin and in its absence. Fig. 2A shows the kinetics of proton efflux from the chromatophore lumen ( $\Delta\text{pH}_{\text{in}}$ ) measured by Neutral red; Fig. 2B shows the kinetics of proton release to the outer medium ( $\Delta\text{pH}_{\text{out}}$ ) measured by Cresol red. Both kinetics roughly matched each other. In the same samples we monitored the  $\Delta\psi$ -changes. The difference kinetic traces reflecting the charge transfer through  $F_0F_1$  (analogous to those shown in Fig. 1E) are plotted as dotted lines in Fig. 2A and B; they follow the respective  $\Delta\text{pH}$ -changes. Hence, the charge transfer through  $F_0F_1$  monitored by carotenoid bandshifts was due to the proton transfer across  $F_0F_1$ . Protons were trapped from the one side of the membrane, transferred across it and then released into the bulk at the other side.

### 3.5. ATP synthesis (Fig. 3A)

The ATP release after one, two and three flashes given at 100 ms interval was detected by luciferin-luciferase luminescence under experimental conditions similar to those of measurements in Figs. 1 and 2. The ATP yield was small after the first flash, larger upon the second one and more so in response to the third flash. After the third, fourth and subsequent flashes the ATP yield per flash remained constant (not documented). Quite expectedly, the luminescence signal attributed here to the ATP synthesis was completely abolished by venturicidin

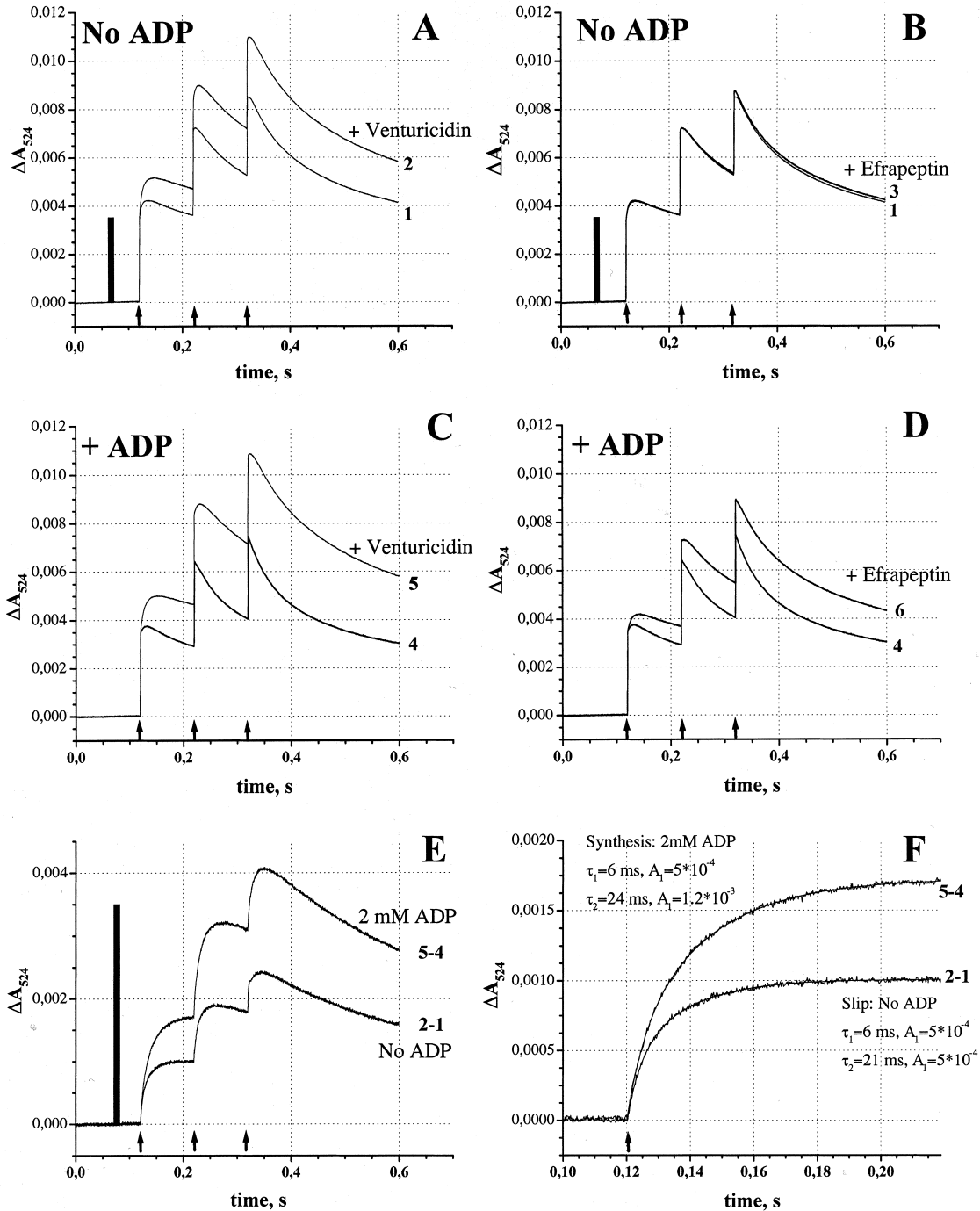


Fig. 1. Charge transfer by  $F_0F_1$ -ATP-synthase of *Rb. capsulatus*. Flash-induced membrane potential changes in the suspension of chromatophores were monitored by the carotenoid bandshift at  $\lambda=524$  nm. Flashes of actinic light are indicated by arrows. See details in text. Panels A and B: 2 mM  $P_i$ , no ADP present; 1, no additions; 2, +200 nM venturicidin; 3, +100 nM efrapeptin. Panels C and D: 2 mM  $P_i$ , 2 mM ADP present; 4, no additions; 5, +200 nM venturicidin; 6, +100 nM efrapeptin. Panel E:  $\pm$  venturicidin difference traces reflecting charge transfer via  $F_0F_1$ -ATP-synthase in the absence (2-1) and in the presence (5-4) of ADP. Panel F: Same curves as in E but on an expanded time scale fitted by two exponential functions. All measurements were done at pH 8.0.

(bottom trace in Fig. 3A), FCCP, valinomycin+ $K^+$ , oligomycin and efrapeptin added in appropriate concentrations (not documented).

### 3.6. Estimation of the $H^+/ATP$ ratio

To calibrate the number of protons transferred by  $F_0F_1$  after each flash we used the fact that the fast, here unresolved, component of  $\Delta\psi$  increase after the first flash (its extent is

shown by a bar in Fig. 1A, B, and E) corresponds to the transfer of a single charge across the membrane by each RC [32]. The RC: $F_0F_1$  stoichiometry was 6:1 in our preparations (see Section 2). As follows from the comparison of data in Figs. 1E and 3A, about 3.0 mol protons were transferred after the first flash per mol ATP-synthase (Fig. 1E, trace 5-4) whereas only 0.23 mol ATP was released (Fig. 3A). This gives an apparent  $H^+/ATP$  ratio of about 13. After three flashes

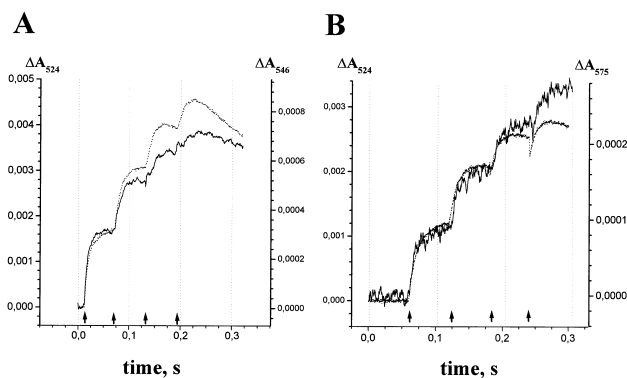


Fig. 2. pH-changes reflecting the charge transfer through the  $F_0F_1$ -ATP-synthase. A: The flash-induced  $\pm$ venturicidin  $pH_{in}$  changes monitored by absorption change of Neutral red ( $\lambda = 546$  nm). B: The flash induced  $\pm$ venturicidin  $pH_{out}$  changes monitored by Cresol red ( $\lambda = 575$  nm). The difference  $\pm$ venturicidin  $\Delta\psi$ -changes measured in same samples are shown by dotted lines. Flashes of actinic light are indicated by arrows. All measurements were done at pH 8.0.

about 8.0 mol protons were transferred yielding 1.6 mol ATP that corresponds to the cumulative  $H^+/ATP$  ratio of about 5.

### 3.7. Dependence of the ATP yield on the ADP concentration (Fig. 3B)

The cumulative ATP yield in response to three sequential light flashes increased gradually upon the elevation of the ADP concentration in the medium. The half-saturation of the effect was achieved at the ADP concentration of 4  $\mu$ M (closed symbols), similar to those causing the half-increase in the carotenoid bandshift decay (open symbols, see also Fig. 1). In the absence of ADP, no ATP release was detected, although the samples were not depleted from the tightly-bound ATP.

## 4. Discussion

We monitored proton transfer by  $F_0F_1$ -ATP-synthase and ATP synthesis in response to a stepwise increasing membrane potential. The ratio between the number of protons translocated by  $F_0F_1$  and the respective ATP yield decreased with each step. The kinetics of proton transfer through  $F_0F_1$  in response to the first potential step contained two components, with approximately 6 ms and 20 ms time constants. Both the extent and the rate of the fast 6 ms component of proton transfer were not dependent on whether ADP was present or not. The extent of this 6 ms component was about one or two protons transferred per one  $F_0F_1$  (the uncertainty is due to the chromatophore heterogeneity discussed in [33]). The extent of the slower 20 ms component was sensitive both to the presence of ADP and to efrapeptin implying that the transfer of the respective protons was coupled with events in  $F_1$ .

The proton transfer by  $F_0$  involves a strictly conserved carboxy group of the  $c$ -subunit (Glu-61 in *Rb. capsulatus*). In *E. coli* these 12 carboxyls on the  $c_{12}$ -ring are pointing outwards [7,8]. In view of these data, the translocation of four protons (needed to synthesize one ATP molecule) in one step is unlikely. Alternatively, it has been suggested that the  $c_{12}$ -ring rotates in such a way that the carboxyls slide along the  $a$ -subunit where they get alternating access to the two bulk phases [2,34]. Protons which are picked from one side are released one by one to the other side of the membrane.

In view of the structural information two plausible mechanisms can account for our data. One formal possibility is that the transition of ATP-synthase from the 'resting' mode into the catalytic one is driven by the transfer of 'activating' proton(s) as discussed in [35,36]. Then the 6 ms component may be attributed to the translocation of activating proton(s)

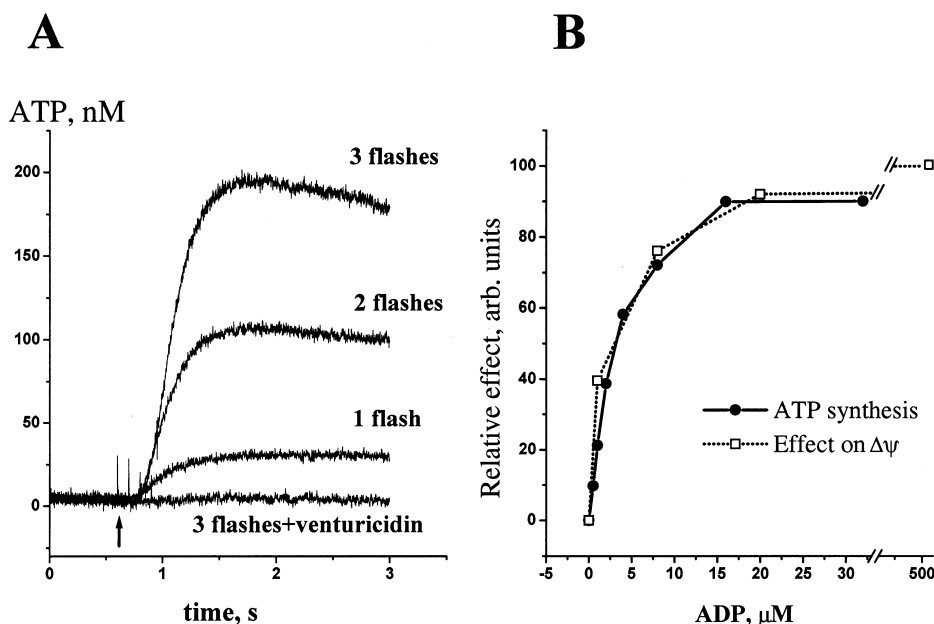


Fig. 3. ATP synthesis driven by the illumination of chromatophores with flashes of light as monitored by luciferin-luciferase luminescence. A: ATP yield after one, two or three flashes given at 100 ms interval and after three flashes but in the presence of venturicidin. B: Dependence of the ATP yield after three flashes (solid symbols, 2 mM  $P_i$  present) and of the increase in  $\Delta\psi$  decay (open symbols, the difference between the  $\Delta\psi$  trace measured in the presence and absence of ADP, respectively, at 200 ms after the third flash) on the ADP concentration in the sample.

whereas the 20 ms component would reflect the following 'coupled' proton flow. The existence of futile 'activating' protons would also be in line with the higher  $H^+/ATP$  ratio in response to the first flash and with the insensitivity of the 6 ms component to the functional state of the  $\alpha_3\beta_3$ -hexagon.

Alternative possibility is that our observations reflect the necessity to store the free energy from the translocation of the first, say 1–3, protons until it could be used, after the translocation of the fourth proton, to power the release of the tightly-bound ATP. An elastic energy storage by a strained conformation of  $F_0F_1$  with  $\gamma$ -subunit acting as a torsional spring has been proposed [2]. As detailed elsewhere (Cherepanov et al., submitted), it is conceivable that the torsional strain is built up in  $30^\circ$  angular steps as driven by each of four subsequent proton transfer events in  $F_0$ . In this case the  $\Delta\mu_{H^+}$ -driven proton translocation, from the first to the fourth step, has to operate against an increasing back pressure. Then the first three protons may be transferred through  $F_1F_0$  without causing ATP release when  $\Delta\mu_{H^+}$  is not high enough to drive the last, fourth proton. This is compatible with our observation that after the first flash about 3 protons were transferred and only 0.23 ATP molecules were released per one ATP-synthase, three times less than one could expect from the  $H^+/ATP$  stoichiometry of 4. The high ATP yield, comparable with the  $H^+/ATP$  stoichiometry of 4 was observed only at higher membrane potential which required three or more flashes in our set-up.

Due to the elasticity, some protons could be translocated through  $F_0F_1$  even in the absence of ADP. This is compatible with experimental observations documented in Figs. 1 and 2. In this framework, the independence of both the extent and the rate of 6 ms component of proton transfer on the presence of ADP (Fig. 1F) may indicate that the straining of  $F_0F_1$  by the initial protons was independent on the functional state of the  $\alpha_3\beta_3$ -hexagon, contrary to the slower transfer of the last protons.

The functionality of mechanic tension has been also claimed by another group, who found that the detachment of  $F_1$  from  $F_0$  was facilitated upon membrane energization [37]. Elasticity of  $F_0F_1$  may result in different averaged conformation of ATP-synthase and ATPase. This may account for the functional inequality of two forms of enzyme noted in the literature [38,39].

The possibility that the transfer of activating proton(s) is followed by stepwise straining of the enzyme driven by the coupled proton flow seems unlikely. Then one could expect more than two kinetic components of proton transfer. This rather favors the attribution of kinetic heterogeneity of proton translocation to the mechanical straining, as the operation of  $F_0F_1$  without transient energy storage is hardly imaginable in the view of structural data.

The data in Figs. 1E and 2 show that proton transfer through  $F_0F_1$  does not stop in the absence of ADP even after several flashes. Hence, the protons, being driven by  $\Delta\mu_{H^+}$ , still find a way to slip through  $F_0F_1$  although no ATP can be synthesized. Previously the phenomenon of proton slip through  $F_0F_1$  was characterized for chloroplast thylakoids [40–42]. In line with observations in [42], the data in Fig. 1 show that the membrane potential threshold for the proton slip was higher than for ATP synthesis. This may effectively prevent the slip under physiological conditions. Mechanically, the proton slip might imply either (i) that the sliding  $c$ -ring

can detach from the  $\gamma$ -subunit or (ii) that the rotation of  $c$ -ring together with  $\gamma$ -subunit could be uncoupled from events in the  $\alpha_3\beta_3$ -hexagon. In the light of the finding that efrapeptin is clamped between the  $\alpha_3\beta_3$ -ring and  $\gamma$ -subunit in the crystal structure [30], the failure to detect the effect of the inhibitor on proton slip (see Fig. 1B) seems to favor a decoupling between  $\gamma$  and the  $c$ -ring. (It is noteworthy, however, that the crystal structure was obtained with adenine nucleotides in the catalytic sites [30] whereas under the slip conditions only the tightly-bound ATP is expected to be present.)

Fig. 3B shows that the increase in the concentration of ADP gradually couples the proton transfer to the ATP synthesis. One possible explanation is the binding change mechanism: the energy of  $\Delta\mu_{H^+}$  may be insufficient to overcome the activation barrier of the ATP release from the tight-binding site unless ADP and  $P_i$  bind to the alternative site. Another plausible explanation is that  $F_1$  may not be in a functional state when only one tightly-bound ATP molecule is present and that the binding of ADP to the alternative site converts the enzyme into the functional conformation. Further studies are necessary to discriminate between these two possibilities.

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