# Chromatophore Vesicles of *Rhodobacter capsulatus* Contain on Average One $F_OF_1$-ATP Synthase Each

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ABSTRACT ATP synthase is a unique rotary machine that uses the transmembrane electrochemical potential difference of proton ($\Delta\tilde{\mu}_{H^+}$) to synthesize ATP from ADP and inorganic phosphate. Charge translocation by the enzyme can be most conveniently followed in chromatophores of phototrophic bacteria (vesicles derived from invaginations of the cytoplasmic membrane). Excitation of chromatophores by a short flash of light generates a step of the proton-motive force, and the charge transfer, which is coupled to ATP synthesis, can be spectrophotometrically monitored by electrochromic absorption transients of intrinsic carotenoids in the coupling membrane. We assessed the average number of functional enzyme molecules per chromatophore vesicle. Kinetic analysis of the electrochromic transients plus/minus specific ATP synthase inhibitors (efrapeptin and venturicidin) showed that the extent of the enzyme-related proton transfer dropped as a function of the inhibitor concentration, whereas the time constant of the proton transfer changed only marginally. Statistical analysis of the kinetic data revealed that the average number of proton-conducting $F_OF_1$-molecules per chromatophore was approximately one. Thereby chromatophores of *Rhodobacter capsulatus* provide a system where the coupling of proton transfer to ATP synthesis can be studied in a single enzyme/single vesicle mode.

## INTRODUCTION

$F_OF_1$-ATP synthase uses the transmembrane electrochemical potential difference of the proton ($\Delta\tilde{\mu}_{H+}$) to synthesize ATP from ADP and inorganic phosphate (for reviews, see Stock et al., 2000; Weber et al., 2000; Boyer, 1998; Junge et al., 1997). This bipartite enzyme is composed of the membrane-embedded ion-translocating $F_O$ and the hydrophilic catalytic $F_1$ that protrudes for more than 100 Å from the plane of the membrane. In bacteria and chloroplasts the $F_O$ part is formed from three different subunits (*a*, *b*, and *c*) in stoichiometry $a_1$:$b_2$:$c_{10-14}$ with *c*-subunits arranged as a ring (Stock et al., 1999; Seelert et al., 2000; Stahlberg et al., 2001). The crystal structure of $F_1$ shows a hexamer of $\alpha_3\beta_3$-subunits with the $\gamma$-subunit as a central shaft (Abrahams et al., 1994) that is connected to subunit $\epsilon$ and the *c*-oligomer (Gibbons et al., 2000; Stock et al., 1999).

ATPase is a rotary machine: the $\gamma_1\epsilon_1c_{10-14}$ complex (the rotor) rotates relative to the other subunits (that form the stator) when driven by the hydrolysis of ATP (Duncan et al., 1995; Sabbert et al., 1996; Noji et al., 1997; Pänke et al., 2000). The mechanism for torque generation by proton flow and its coupling to ATP synthesis has been discussed (e.g., in Junge et al., 1997; Dimroth et al., 1999; Wang and Oster, 1998; Cherepanov et al., 1999). The common features of the proposed mechanisms are 1) a ring of *c*-subunits, each of them carrying one carboxy residue capable of proton binding; 2) the alternating accessibility of this proton binding residue from different sides of the membrane; 3) Brownian rotation of this ring relative to $ab_2$ subunit complex; and 4) electrostatic constraints enforcing the sequential deprotonation/reprotonation of the acidic residue on the *c*-subunit depending on its position relative to the subunit *a* and the lipid phase. In some organisms proton is substituted by sodium (Dimroth, 2000).

Preparations of chromatophores (vesicles derived from invaginations of the cytoplasmic membrane) of phototrophic bacterium *Rhodobacter capsulatus* proved to be convenient for investigation of the ion-conducting properties of the ATP synthase. Excitation of chromatophores with short flashes of light generates steps of protonmotive force. Its chemical component ($\Delta$pH) has been first monitored by absorption transients of pH-indicating dyes (Jackson and Crofts, 1969a) and calibrated by the amphiphillic dye neutral red (Mulkidjanian and Junge, 1994). The electrical component ($\Delta\psi$) has been monitored and calibrated by intrinsic electrochromic bandshifts of carotenoids at 520 nm (Jackson and Crofts, 1969b) Proton transfer across the coupling membrane, driving the synthesis of ATP, has been apparent from an accelerated decay of the electrochromic absorption transients (Jackson et al., 1975). It is sensitive to specific inhibitors of $F_1$ (efrapeptin) and $F_O$ (venturicidin, DCCD, oligomycin). The charge transfer has been calibrated and correlated with the ATP yield, which has been measured in the same samples by the luciferin-luciferase system (Saphon et al., 1975; Feniouk et al., 1999, 2001).

In this work we addressed the question of the average number of the $F_OF_1$-ATP synthases per chromatophore ves-

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icle. We obtained experimental evidence that routine preparations of chromatophores from *Rb. capsulatus* contained, on the average, approximately one molecule of ATP synthase per vesicle. This finding greatly reduced the statistical ambiguity over active/inactive vesicles or enzymes and paved the way for studying the operation of the ATP synthase in a single enzyme/single vesicle mode.

## MATERIALS AND METHODS

### Cell growth and chromatophore preparation

Cells of *Rb. capsulatus* (wild type, strain B10) were grown photoheterotrophically on malate as a carbon source at +30°C (Lascelles, 1959). The bottles were illuminated with four Osram L 36W/25 and three Osram L 18W/21-840 candle-shaped bulbs. The average light intensity was 18 $W/m^2$. Cells were harvested at the end of logarithmic growth phase, if not otherwise indicated; the cell mass of the culture was estimated by measuring optical density at 660 nm (Schumacher and Drews, 1979).

Harvested cells were washed twice with 30 mM HEPES-KOH (pH 7.4), 5 mM $MgCl_2$, 0.5 mM dithiothreitol, 50 mM KCl, 10% sucrose, and resuspended in the same pH buffer. A few flakes of DNase were added. The cells were disrupted by sonication on ice (Branson Sonifier B15, four or five exposures for 15 s with 1-min incubation in between), and centrifuged (20,000 × *g*, 20 min, 4°C) to remove large cell fragments. The pellet was suspended in the same buffer and resonicated as above; the resonicated suspension was centrifuged again (20,000 × *g*, 20 min, 4°C). Supernatants were collected and centrifuged (180,000 × *g*, 90 min, +4°C). The pellet was resuspended in 30 mM HEPES-KOH (pH 7.4), 5 mM $MgCl_2$, 0.5 mM dithiothreitol, 50 mM KCl, 20% sucrose. It contained chromatophores at a bacteriochlorophyll concentration of 0.3 to 0.7 mM. Chromatophores were stored at −80°C until use.

The concentration of bacteriochlorophyll in the samples was determined spectrophotometrically in the acetone-methanol extract at 772 nm according to Clayton (1963). The amount of functionally active reaction centers (RC)s was estimated from the extent of flash-induced absorption changes at 603 nm (as in Mulkidjanian et al., 1991).

### Preparation of chromatophores stripped of $F_1$

Chromatophores were depleted of $F_1$ by EDTA-treatment (Melandri et al., 1970,1971; Baccarini-Melandri et al., 1970). Chromatophores from frozen stock were thawed and diluted 25-fold with 1 mM EDTA, pH 8, in daylight. The suspension was sonicated 5 times for 20 s with 1-min interval (on ice) and then centrifuged at 180,000 × *g*, 90 min, +4°C. The pellet was resuspended in a medium containing 20 mM HEPES KOH (pH 7.4), 5 mM $MgCl_2$, 0.5 mM dithiothreitol, 50 mM KCl to yield a bacteriochlorophyll concentration of 0.3 to 0.7 mM and used on the same day or after storage at +4°C overnight.

### Spectrophotometric measurements

The kinetic flash-spectrophotometer was constructed according to Junge (1976). Monitoring light was provided by a halogen 200-W lamp, it was heat-filtered (KG 2 filter, SCHOTT, Mainz, Germany) and passed through a 11-nm wide interference filter peaking at 522 nm (SCHOTT). A shutter, placed in front of the cuvette, eliminated the actinic effect of the monitoring beam between the measurements. The dead time between the opening of the shutter and the actinic flash was 400 ms. Changes in transmitted light intensity ($\Delta I$) were monitored by a photomultiplier (9801B, Thorn EMI, Ruislip, UK) that was shielded from the actinic flash with two blue filters (BG 39/2, SCHOTT). The DC output from the photomultiplier was preset to 1 V (load resistor: 10 kΩ) by varying the output current of the photomultiplier power supply. The photomultiplier output was connected to the positive input of a difference amplifier (AM502, Tektronix, Beaverton, OR). Before an actinic flash was fired, the signal was sampled and held by a homemade amplifier (N. Spreckelmeier) connected to the negative input of the difference amplifier. The difference signal was amplified 100-fold, digitized, and stored on an averaging oscilloscope (Pro 10, Gould Nicolet, Erlensee, Germany). The analogue bandwidth was 3 kHz and the digital time-per-address was 200 μs. The optical path was 1 cm, both for the exciting and for the measuring beam. The final concentration of bacteriochlorophyll in the cuvette was 8 to 12 μM.

Eight signals measured at a repetition rate of 0.08 Hz were averaged. The dark adaptation time of 12 s between flashes was chosen to allow for the complete decay of the transmembrane electrical potential difference ($\Delta\psi$). This time interval was still short enough to prevent the deactivation of the $F_OF_1$-ATP synthase. (The expected life time of the $\Delta\psi$-activated state of the $F_OF_1$ ATP-synthase under these conditions was 30–70 s (Turina et al., 1992).)

Electrochromic absorption transients at 522 nm were used to monitor the transmembrane voltage (Clark and Jackson, 1981; Symons et al., 1977; Jackson and Crofts, 1971).

Saturating actinic flashes were provided by a xenon flash lamp (full width half maximum ~10 μs, red optical filter (RG 780, SCHOTT)). The energy density on the cuvette was 12 $mJ/cm^2$). Excitation by a xenon flash gave 8 to 11% greater signals than by the laser pulse (duration < 100 ns). It was indicative of double turnover in ~10% of RCs, in agreement with the recent report on the presence of a 3-μs component in the reduction of $Q_B$ in chromatophores of *Rb. sphaeroides* and *Rb. capsulatus* (Tiede et al., 1998).

### Incubation medium

Chromatophores were suspended in a medium that contained 50 mM KCl, 0.3% bovine serum albumin, 5 mM $MgCl_2$, 200 μM succinate/2 mM fumarate (redox buffer), 5 or 10 μM 1,1′-dimethylferrocene (redox mediator), and 2 mM KCN (to block the terminal oxidase). Instead of succinate-fumarate redox system, 2 mM $K_4Fe[CN]_6$ was present in some experiments.

## RESULTS AND DISCUSSION

Fig. 1 *A* illustrates typical absorption changes at 522 nm in chromatophores of *Rb. capsulatus* in response to two saturating flashes of light given at 240-ms interval. These changes reflect mainly electrochromic bandshifts of carotenoids and can be described by the following expression:

$$\Delta A = \sum a_i \Delta A_i + b; \qquad i = 0, 1, 2, \ldots$$

in which $\Delta A_i$ is the absorption change proportional to the transient transmembrane voltage change ($\Delta\psi_i$) in the subset of chromatophore vesicles containing *i* molecules of $F_OF_1$, $a_i$ is the weight of this subset, and *b* is a residual flash-induced absorption change that is unrelated to the delocalized transmembrane voltage (local electrochromic effects, absorption transients of the primary electron donor of the photochemical reaction centers, etc). Under the chosen conditions the relative extent of *b* was less than 10%. Its magnitude was checked by addition of $K^+$ + valinomycin to rapidly quench the transmembrane voltage (data not shown). Therefore, to a first approximation the absorption

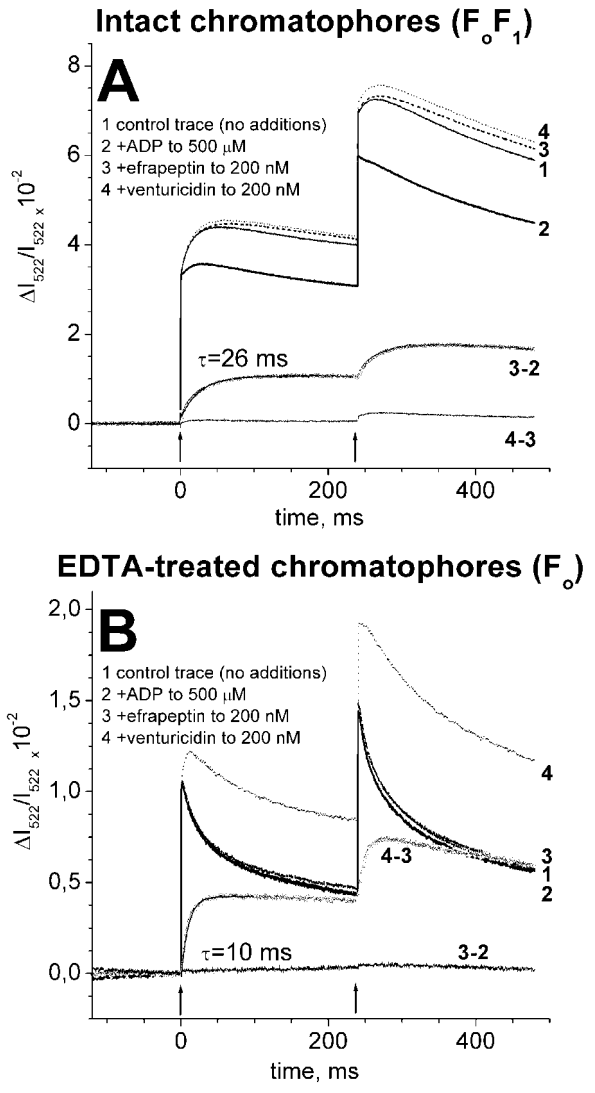


FIGURE 1 Flash-induced absorption changes at 522 nm in a suspension of *Rb. capsulatus* chromatophores. The supending medium contained 2 mM $K_2HPO_4$, 2 mM $K_4[Fe(CN)_6]$, 5 $\mu$M 1,1′-dimethylferrocene, 2 mM KCN, 5 mM $MgCl_2$, 50 mM KCl, 0.3% bovine serum albumin, pH 7.9. (Trace 1) Control, no additions; the following compounds were added consecutively to the sample: ADP, 500 $\mu$M (trace 2), efrapeptin; 200 nM (trace 3), venturicidin, 200 nM (trace 4). Difference traces reflect proton flow through $F_O(F_1)$ (3-2) and $F_O$ (4-3), respectively. Actinic flashes are marked by arrows. See details in text. (*A*) Well-coupled chromatophores. (*B*) Chromatophores depleted of $F_1$ by EDTA treatment.

changes at 522 nm represent the weighted sum of the transmembrane voltage transients in a heterogeneous ensemble of chromatophore vesicles containing different copy numbers of ATP synthase.

Each flash caused a sharp, here unresolved rise of the absorption at 522 nm. It was attributable to the charge separation in the photochemical RC. It was followed by a slower rise due to charge transfer in the cytochrome $bc_1$-complex (for reviews on the RC and the cytochrome $bc_1$-complex, see Jackson, 1988; Crofts and Wraight, 1983).

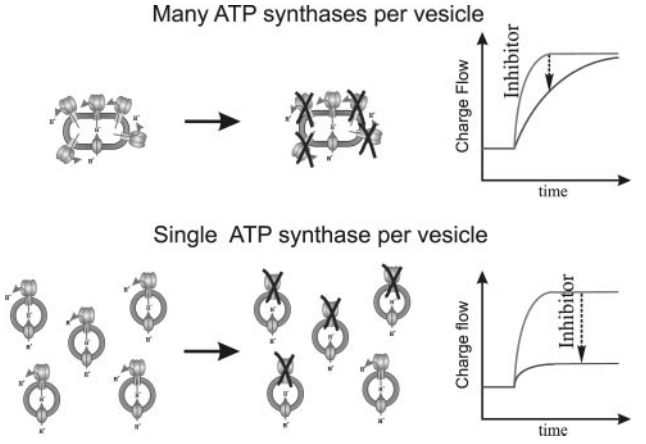


FIGURE 2 Schematic representation of the inhibitor titration of electrochromic absorption transients under two different assumptions on the ATP synthase-contents in chromatophores (see text for details).

The biphasic rise was followed by a multiphasic decay that was markedly accelerated by the addition of ADP and inorganic phosphate (trace 1 and 2 in Fig. 1 *A*). Similar observations have been made in chromatophores of *Rb. sphaeroides* (Jackson et al., 1975; Petty and Jackson, 1979). The effect of ADP and phosphate was reversed by efrapeptin, a peptide inhibitor that binds between subunit $\gamma$ and $\alpha_3\beta_3$ hexamer in $F_1$ (Abrahams et al., 1996). The inhibitor decreased the decay rate almost to the level that was observed in the absence of ADP and phosphate (Fig. 2 *A*, trace 3). Venturicidin, oligomycin, and DCCD, all inhibitors of proton conduction through the $F_O$-portion of ATP synthase (von Brufani et al., 1968; Linnett and Beechey, 1979), decreased the rate of the decay further, even in the absence of ADP or phosphate or in the presence of efrapeptin (Fig. 1, *A* and *B*, trace 4). As discussed elsewhere (Feniouk et al., 1999, 2001), the greater efficiency of $F_O$-inhibitors as compared with the $F_1$-inhibitor efrapeptin is due to the fact that the former blocks the proton conductivity at $F_O$, independent of the presence/absence or functionality of $F_1$, whereas efrapeptin acts only on $F_OF_1$ via $F_1$ proper. When chromatophores were stripped of $F_1$ by EDTA-treatment as in Baccarini-Melandri and Melandri (1971), the decay of the electrochromic transient lost its sensitivity to ADP+$P_i$ or efrapeptin, as expected, but it was still sensitive to venturicidin (Fig. 1 *B*). It was obvious from Fig. 1 *B*, that the faster decay of the electrochromic transient was limited in extent. A substantial slowly decaying level remained even 200 ms after the flash. This held true, both in well coupled and in $F_1$-stripped chromatophores. This residual level was markedly higher after the second flash. This behavior suggested the existence of a subset of chromatophores with very low conductivity because of the total lack of $F_O(F_1)$. (Hereafter we denote by $F_O(F_1)$ the proton-conducting, and venturicidin-sensitive entity, which is either bare $F_O$ or whole $F_OF_1$ (coupled or slipping).) Excitation of this subset of chro-

matophores by repetitive light flashes led to a stepwise increase of the electrochromic transient. The presence of a $F_O(F_1)$-lacking fraction of chromatophores is in agreement with the previous observations that the removal of $F_1$ by EDTA treatment did not result in a fast and complete dissipation of $\Delta\psi$ and $\Delta$pH to the zero level after an actinic flash (Saphon et al., 1975; Melandri et al., 1970, 1972). The traces presented in Fig. 1 *B* allow to roughly estimate the relative fraction of such $F_O(F_1)$-lacking chromatophores as ~50%. A detailed analysis of kinetic traces, which has been presented elsewhere (Feniouk et al., 2001) yielded a somewhat lower estimate of 40%.

We determined the average amount of $F_O(F_1)$ per vesicle and asked for the homogeneity of the $F_O(F_1)$ distribution over the ensemble of chromatophores. The observed rapid relaxation of the electrochromic absorption transients reflects the superimposition of events in many vesicles with different surface densities of $F_O(F_1)$. We used an approach similar to one developed elsewhere (Schmid and Junge, 1975; Drachev et al., 1981; Lill et al., 1987) to investigate the distribution of $F_O(F_1)$ in chromatophores. The scheme in Fig. 2 illustrates how the titration with an $F_O$ inhibitor reveals the number of ion transporter molecules per vesicle. If several ATP synthases were present per vesicle, the inhibition of a few would only slow down the electric relaxation but not decrease its extent (unless the number of conducting $F_O(F_1)$ was reduced to zero). Alternatively, if most of the vesicles contained none or just one single enzyme molecule, then the latter subset of vesicles caused an all-or-none response to the binding of the inhibitor. When the inhibitor concentration was increased, more and more of such units were switched off, and the extent of the rapid relaxation decreased. Fig. 3, *A* through *C* shows the results of a titration with efrapeptin of well-coupled chromatophores and Fig. 3, *D* through *F* the titration with venturicidin of $F_1$-depleted chromatophores. In both cases the electrochromic transients recorded in the completely inhibited sample were subtracted from those recorded in the presence of subsaturating, gradually increasing inhibitor concentrations. It resulted in a series of kinetic traces of proton transfer attributable to a decreasing fraction of active enzyme molecules (Fig. 3, *B* and *E*). In both cases, the extent of the rapid proton transfer dropped as a function of the inhibitor concentration, whereas the time constant of the proton transfer was only marginally changed (Fig. 3, *C* and *F*). According to the above rationale the results were qualitatively in line with the assumption that the average number of proton-conducting $F_O(F_1)$ per vesicle was close to 1.0.

The exact number was calculated using the statistical model that was developed for treating similar questions in isolated thylakoids from higher plants (Schmid and Junge, 1975; Lill et al., 1987). The model has been based on three assumptions: 1) the distribution of vesicles over their membrane area is narrow; 2) the number of active conducting entities ($F_O(F_1)$ in the present case) per chromatophore obeys Poisson's distribution,

$$P(n) = \frac{\bar{n}^n}{n!}\exp(-\bar{n}); \tag{1}$$

and 3) the voltage decay in a given vesicle with $n$ channels is monoexponential with a rate constant proportional to $n$:

$$U(n, t) = U_0\exp(-n \times k_0 \times t),$$

in which $k_0$ is the decay rate in a vesicle with a single molecule of ATP synthase and $U_0$ is the amplitude of the voltage change. The apparent decay of the transmembrane voltage in the entire ensemble of chromatophores is then described by the equation (Lill et al., 1987):

$$U_{app}(t) = U_0 \times \exp(-\bar{n}) \times \exp(\bar{n} \times \exp(-k_0 \times t)) \tag{2}$$

in which $\bar{n}$ is the average number of channels per vesicle. The latter parameter can be experimentally controlled by the addition of an appropriate inhibitor. The interaction of the inhibitor with $F_O(F_1)$ is described by a single equilibrium constant $K_I$ so that

$$[EI] = K_I \times [E] \times [I] \tag{3}$$

in which [E] is the concentration of the free (active) enzyme, [I] is the concentration of free inhibitor, and [EI] is the concentration of the inhibited (inactive) enzyme. The mass balance requires that

$$[E] + [EI] = [E]_{total} \tag{4}$$

$$[I] + [EI] = [I]_{total} \tag{5}$$

where $[E]_{total}$ and $[I]_{total}$ are the total concentrations of the enzyme and the inhibitor, respectively. Eqs. 3 to 5 yield the concentration of the active form of $F_O(F_1)$:

$$[E] = \tfrac{1}{2}\left[\sqrt{([I]_{total} - [E]_{total} + 1/K_I)^2 + 4[E]_{total}/K_I}\right] - [I]_{total} + [E]_{total} - 1/K_I \tag{6}$$

The average number of uninhibited ATP synthase molecules per chromatophore, $\bar{n}$, as function of the total inhibitor concentration is thereby

$$\bar{n} = \overline{n_0} \times [E]/[E]_{total} \tag{7}$$

in which $\overline{n_0}$ is the average number of active $F_O(F_1)$ molecules per chromatophore before the addition of the inhibitor.

Thus, the complete model includes five fit parameters: the initial extent of the voltage, $U_0$, the decay rate through a single channel, $k_0$, the total concentration of proton-conducting $F_O(F_1)$ molecules, $[E]_{total}$, the average number of enzyme molecules per chromatophore, $\overline{n_0}$, and the inhibition constant, $K_I$.

Two sets of kinetic traces of charge transfer through the coupled and uncoupled ATP synthase obtained at different

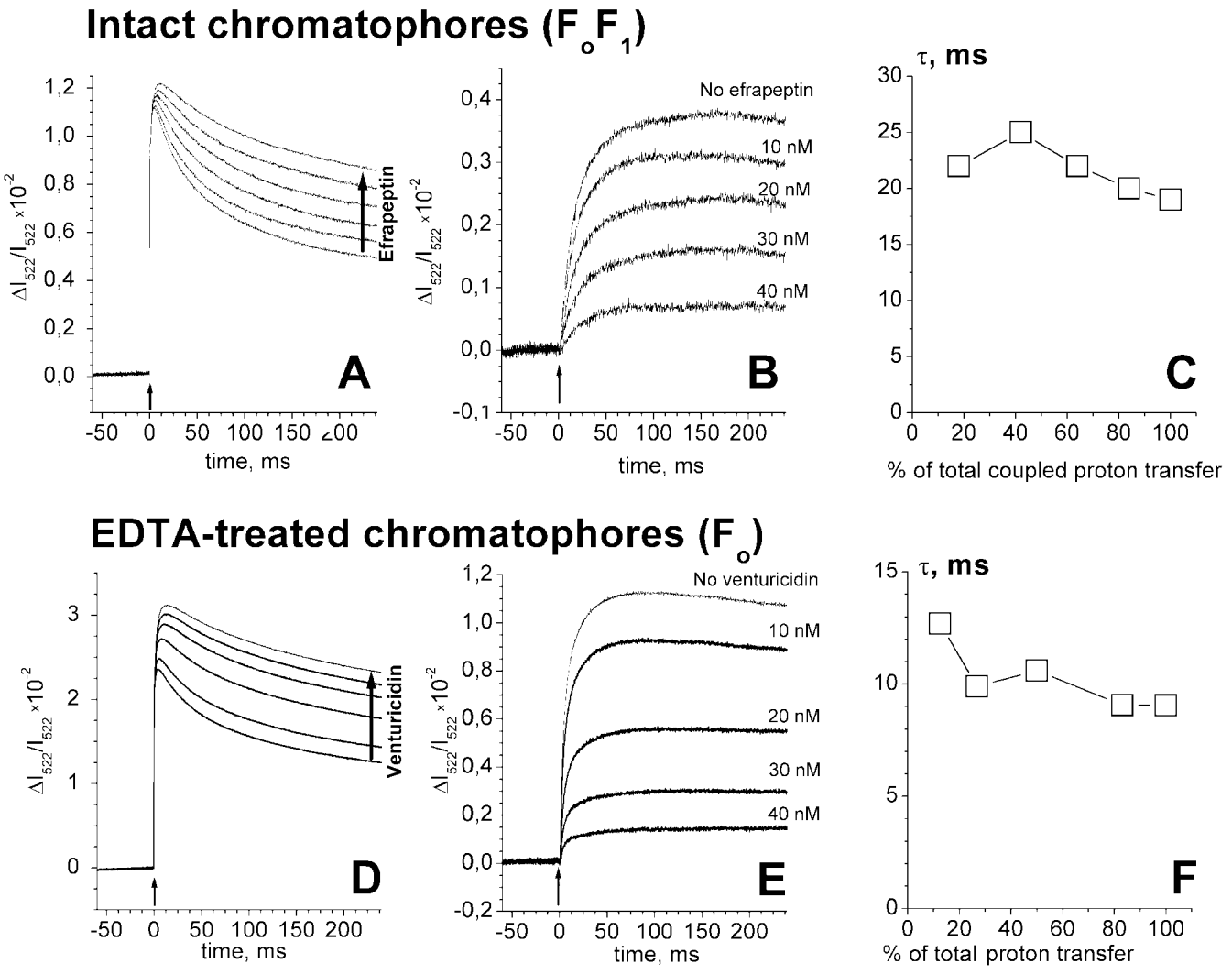


FIGURE 3 Titration by efrapeptin and venturicidin of electrochromic absorption transients through $F_O(F_1)$ in intact (*A–C*) and uncoupled (*D–F*) chromatophores of *Rb. capsulatus*, respectively. Actinic flashes are marked by arrows. (*A* and *D*) The increase in the inhibitor concentration slowed the decay rate. (B and E) Difference traces reflecting proton transfer through $F_O(F_1)$ at subsaturating inhibitor concentrations (see text); (*C* and *F*) the relaxation time of proton transfer through $F_O(F_1)$ as function of the inhibitor concentration.

concentrations of efrapeptin and venturicidin are represented in Fig. 3, *B* and *E*, respectively. We used numerical optimization to describe these traces by the model. The found parameter values are listed in Table 1. The experimental and the calculated voltage decay curves as function of inhibitor concentration are shown in Fig. 4 *A* (titration by efrapeptin) and in Fig. 4 *B* (titration by venturicidin), respectively. Titrations by either inhibitor gave the same result: each chromatophore contained 1 molecule of $F_O(F_1)$ on the average.

The results of the inhibitor titration were independent on whether chromatophores were prepared by sonication or by a French-Press treatment (data not shown). Routinely chromatophores were prepared from cells that were harvested at

**TABLE 1 Parameters for the fit by Eq. 2 of the $F_o(F_1)$-related decay of electrochromic absorption transients under titration with efrapeptin and venturicidin, respectively**

| | $[E]_{total}$, nM | $K_I$, $nM^{-1}$ | $k_0$, $s^{-1}$ | $U_0$, charge/RC | $\overline{n_0}$ |
|---|---|---|---|---|---|
| Efrapeptin titration | 38.8 ± 8.2 | 1.12 ± 0.01 | 39 ± 20 | 0.54 ± 0.03 | 1.14 ± 0.06 |
| Venturicidin titration | 28.4 ± 5.9 | 0.760 ± 0.008 | 135 ± 69 | 0.80 ± 0.04 | 1.01 ± 0.05 |

Standard deviations were calculated by the Hessian matrix used in the nonlinear least squares analysis (see e.g. (Bevington, 1969)).

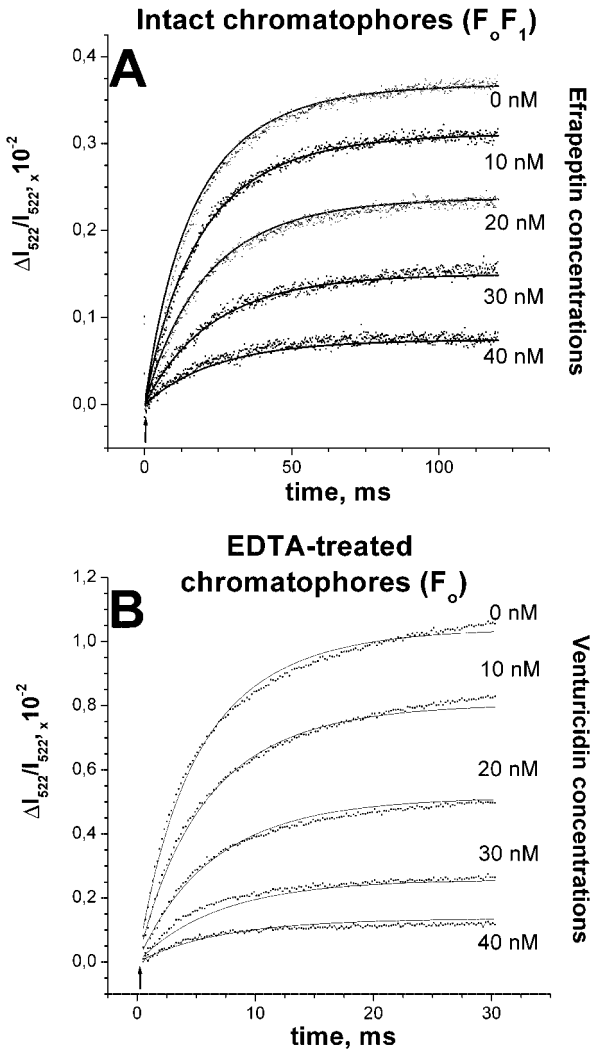


FIGURE 4 Fit of the difference traces presented in Fig. 3, *B* and *E* by the statistical model based on Poisson's distribution (see Eq. 2 and Lill et al., 1987). Fitting parameters are given in Table 1. See text for details.

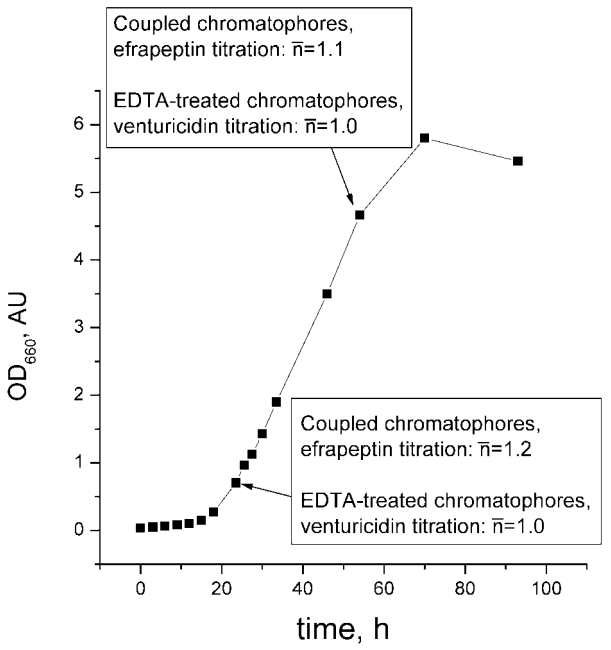


FIGURE 5 Growth curve of *Rb. capsulatus* cells as derived from the absorption of cell suspension at 660 nm. The average amount of ATP synthase molecules per chromatophore vesicle, as determined by the efrapeptin and venturicidin titrations, at different stages of *Rb. capsulatus* cell growth, is indicated.

the late exponential phase of their growth. We checked whether the average number of the ATP synthases per chromatophore depends on the growth stage of the cells. Chromatophores were isolated from cells that were harvested at an early growth stage (for the growth curve, see Fig. 5) and inhibitor titrations by efrapeptin and venturicidin (after EDTA treatment) were carried out as above. The results of the statistical analysis are presented in Fig. 5. The average number of ATP synthase molecules per vesicle was as small as at the end of the growth phase.

Our own electron microscopy data on chromatophores from *Rb. capsulatus* (data not documented) were similar to published EM pictures of chromatophores from *Rb. capsulatus* and *Rb. sphaeroides* showing very few of mushroom-like structures, i.e., $F_OF_1$ on the chromatophore surface (Yen et al., 1982), in contrast with the inner mitochondrial membrane, that is functionally homologous to the chromatophore membrane, but is densely covered with "mushroom-like" ATP synthase complexes (see, e.g., Tsuprun et al., 1989). The finding that only one copy of $F_OF_1$ is present in most of the chromatophore vesicles might be attributed to the smallness of chromatophores (typical diameter 30 to 60 nm) and the necessity to house large amounts of the light-harvesting complexes in the membrane.

For $\overline{n_0} = 1$, the fraction of chromatophores without $F_O(F_1)$ can be calculated from Poisson's distribution as 37%. This value is pretty close to the estimate of 40% that has been previously obtained based on a different approach (see Discussion above and Feniouk et al., 2001). This similarity indicates that the $F_O(F_1)$ distribution among chromatophore vesicles is nearly homogeneous. Another supporting evidence is the value of $K_i$ for venturicidin, which we obtained from titrations of the $F_O$-related proton flux extent in of $F_1$-stripped chromatophores with this inhibitor. Assuming that one molecule of inhibitor per enzyme was sufficient for complete inhibition, we obtained a value of $K_i = 0.41 \pm 0.1\ nM^{-1}$ (data not shown), which was quite close to the $K_I$ value given in Table 1.

It is noteworthy that $\overline{n_0} = 1$ implies that 37% of chromatophores carry zero, 37% one, 18% two, and 6% three proton-conducting enzyme molecules (Fig. 6, upper box). The electrochromic relaxation is then multiphasic. For conduction studies it is, of course, desirable to eliminate the statistical spread and to obtain a "digital" behavior, where almost all vesicles fall in either of two classes with zero or

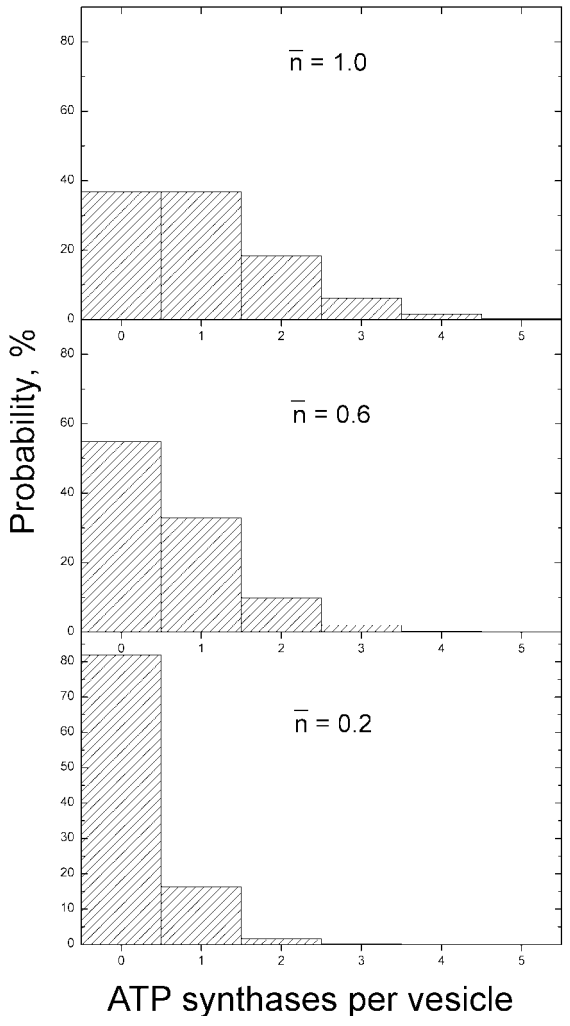


FIGURE 6 Schematic illustration to Poisson's distribution of ATP synthase molecules in chromatophore vesicles. The upper box holds for chromatophore suspension in the absence of inhibitor of proton conduction by $F_O(F_1)$, middle and bottom boxes illustrate partially inhibited samples.

only one proton-conducting molecule. This can be achieved by blocking some of $F_O(F_1)$, e.g., by venturicidin. On the one hand, it is disadvantageous that the proportion of non-conducting vesicles is thereby increased, but on the other hand, the ratio between vesicles with one single conducting molecule and those with more than one increases. With 40% of the enzymes blocked ($\overline{n_0} = 0.6$), the ratio between proton-conducting chromatophores with $n = 1$ over those with $n > 1$ amounts to 2.7 with 80% blocked the ratio increases to 9.3. Under these conditions the subset of vesicles with more than one proton-conducting molecule is negligible. Despite the inhibition of a certain fraction of proton conducting molecules the signal-to-noise ratio is still high enough for single-molecule/single vesicle studies on ion conduction by $F_O(F_1)$ (Figs. 3 and 4). The common crux in enzyme kinetics, namely the ambiguity between reduced turnover rate and inactivation, is here "automatically" solved. This ambiguity is particularly inconvenient considering site-specific mutations, which can affect the enzyme turnover rate and/or the enzyme assembly in the membrane.

We intend to exploit the technique described to investigate proton transfer through $F_O$ and $F_OF_1$ in chromatophores of mutant strains of *Rb. capsulatus*. A proton conductor, like ATP synthase has a single-channel conductance of a few femto-Siemens, which is by orders of magnitude too low to be detected by patch-clamp technique. Whether the method described above can be extended to other ion-translocating enzymes with single-channel conductance in the range of femto-Siemens is an interesting possibility that we are currently investigating.

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