Stepwise rotation of the γ-subunit of EF_0F_1-ATP synthase observed by intramolecular single-molecule fluorescence resonance energy transfer[1]

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Abstract The EF_0F_1-ATP synthase mutants bQ64C and γT106C were labelled selectively with the fluorophores tetramethylrhodamine (TMR) at the b-subunit and with a cyanine (Cy5) at the γ-subunit. After reconstitution into liposomes, these double-labelled enzymes catalyzed ATP synthesis at a rate of 33 s^{-1}. Fluorescence of TMR and Cy5 was measured with a confocal set-up for single-molecule detection. Photon bursts were detected, when liposomes containing one enzyme traversed the confocal volume. Three states with different fluorescence resonance energy transfer (FRET) efficiencies were observed. In the presence of ATP, repeating sequences of those three FRET-states were identified, indicating stepwise rotation of the γ-subunit of EF_0F_1.

Key words: H^+-ATP synthase; EF_0F_1; Inter-subunit rotation; Single-molecule fluorescence resonance energy transfer

1. Introduction

Membrane-bound H^+-ATP synthases catalyze the formation of ATP from ADP and inorganic phosphate in mitochondria, chloroplasts and bacteria. Endergonic ATP synthesis is coupled to proton translocation across the membrane. The enzyme consists of two major parts. The hydrophobic membrane-integrated F_0 part is involved in proton transport across the membrane, whereas the hydrophilic F_1 part contains nucleotide and phosphate binding sites. In *Escherichia coli*, the F_1 part consists of five different subunits with the stoichiometry $\alpha_3\beta_3\gamma\delta\varepsilon$ and the F_0 part is built of three different subunits with a likely stoichiometry ab_2c_{10-14} [1]. The three catalytic nucleotide binding sites on the β-subunits undergo conformational changes [2,3], adopting in sequential order the 'open', 'tight' and 'loose' conformation during ATP-synthesis [4]. These changes are induced by 'docking–undocking' steps of the γ-subunit to the three different αβ-pairs, i.e. by a rotation of the γ-subunit (for reviews, see [4–6]).

ATP-induced rotation of the γ-subunit within the $\alpha_3\beta_3$ barrel has been deduced from ensemble measurements [7,8] and was convincingly visualized by a single-enzyme approach [9]. In these experiments, the $\alpha_3\beta_3\gamma$-fragment of the H^+-ATPase from *Bacillus* PS3 was attached with three His-tags onto the glass surface of a microscopic coverslip, and, as an indicator, a fluorescent μm-long actin filament was connected to the γ-subunit. The orientation of the actin filament with respect to the $\alpha_3\beta_3$ hexamer was monitored by videomicroscopy. At very low ATP concentrations, rotation occurred in discrete 120° steps consistent with sequential ATP hydrolysis at the three β-subunits [10]. Recently, substeps during ATP hydrolysis have been identified [11]. Rotation of the γ-subunit was shown by the fluorescent actin filament method with F_1 parts from chloroplasts and *E. coli* [6] and was used to demonstrate rotation of the ε-subunit and the c-ring in F_0F_1 [12,13]. According to these results, subunits $\gamma\varepsilon c_{10-14}$ ('rotor') rotate against subunits $\alpha_3\beta_3\delta ab_2$ ('stator') counter-clockwise during ATP hydrolysis, when viewed from the membrane to F_1. However, it was not possible to demonstrate with this method that proton transport is coupled with the movement of the rotor subunits.

To ensure coupling during ATP hydrolysis and to maintain full enzymatic activity for ATP synthesis, we incorporated the holoenzyme F_0F_1 into a lipid membrane and labelled specifically the γ-subunit and b-subunit with two different fluorophores chosen for efficient fluorescence resonance energy transfer (FRET). These indicator molecules were small enough to allow for an unconstrained subunit movement. Intramolecular subunit movement resulting in a change in the distances between the fluorophores were detected by changes in FRET efficiencies. After reconstitution of the labelled F_0F_1 into liposomes, ATP synthesis was observed by generating a pH difference and an additional electric potential difference across the membrane. Using confocal single-molecule fluorescence detection, we measured subunit movements during ATP hydrolysis by intramolecular FRET in fully functional single EF_0F_1 reconstituted in liposomes.

2. Materials and methods

The plasmid pRA114 carrying the γT106C mutation was constructed by Aggeler and Capaldi [14] and was expressed in strain RA1 [15]. EF_1 was isolated according to [16]. The γ-subunit of EF_1 was selectively labelled with carbocyanine dye Cy5®-maleimide in 50 mM MOPS/HCl (pH 7.0) as described previously [17,18]. Cy5-malei-

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[1] This paper is dedicated to Prof. H.T. Witt on the occasion of his 80th birthday.

Abbreviations: FRET, fluorescence resonance energy transfer; AMPPNP, adenosine-5′-(β,γ-imido)triphosphate; Cy5, carbocyanine dye Cy5®; TMR, tetramethylrhodamine

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mide was kindly provided by E. Schweinberger and C.A.M. Seidel (MPI für Biophysikalische Chemie, Göttingen, Germany). EF_1 concentrations were determined by UV absorption using an extinction coefficient $\varepsilon = 191\,000\ M^{-1}\ cm^{-1}$ at 280 nm. For Cy5, an extinction coefficient of $250\,000\ M^{-1}\ cm^{-1}$ at 649 nm was used. Unbound dye was removed after 4 min by passing twice through Sephadex G50 centrifuge columns. Specificity of γ-subunit labelling was checked by fluorescence detection after SDS–PAGE. Approximately 65% of EF_1 was labelled with Cy5. EF_1-γT106C-Cy5 was stored at −80°.

In order to introduce a cysteine in the b-subunit, the new plasmid pRR76/bC21S/bQ64C was constructed from pRA100 [19] with pNOC/bC21S obtained from R. Fillingame [20]. The mutation bQ64C was introduced into pNOC by a two-stage PCR mutagenesis procedure [21] to yield pRR36. The fragment with the two mutations was ligated to pRA100. The resulting plasmid pRR76 was expressed in strain RA1 [15]. EF_0F_1 was isolated according to [22]. The b-subunits in this EF_0F_1 were labelled with tetramethylrhodamine (TMR)-maleimide (Molecular Probes) as described above. The degree of labelling was adjusted to approximately 20% to avoid labelling of both b-subunits at the same enzyme. EF_0F_1-bQ64C-TMR was reconstituted in liposomes [22] with a stoichiometry of one EF_0F_1 per liposome. The F_1 parts were removed [23] and the liposomes containing EF_0-bQ64C-TMR were reassembled with EF_1-γT106C-Cy5 to yield liposomes with the double-labelled enzyme EF_0-bQ64C-TMR-F_1-γT106C-Cy5 according to [24,25]. The efficiencies of removal of F_1 and reassembling with labelled F_1 were controlled by measuring ATP synthesis activities. The initial rate of ATP synthesis catalyzed by EF_0F_1 was measured after an acid–base transition at 23°C as described previously [22].

Single-molecule fluorescence measurements of EF_0F_1 in liposomes were performed with a confocal set-up of local design [25]. A frequency doubled Nd:YAG laser (532 nm, 50 mW, Coherent, Germany) was used for excitation. The laser beam was attenuated to 120 μW and focussed by a water immersion objective (UAPO 40×, N.A. 1.15, Olympus) into the buffer solution placed on a microscope slide. Fluorescence was measured in two spectral regions. Single photons were detected with avalanche photodiodes (SPCM-AQR-15, EG&G, Canada) after passing an interference filter HQ 575nm/65nm for TMR and a HQ 665nm LP for Cy5 (AHF, Germany). Photons in two channels were counted in parallel by a multichannel scaler (PMS 300, Becker & Hickl, Berlin, Germany). The actual confocal detection volume $V = 7.7$ fl was calculated from fluorescence correlation spectroscopy using rhodamine 6G according to [18]. All fluorescence measurements were performed in buffer A (50 mM HEPES/NaOH, 2.5 mM $MgCl_2$, 400 μM sodium ascorbate, pH 8.0). Fluorescent impurities of the buffer were removed by activated charcoal granula (Merck, Germany) with subsequent sterile filtration. ATP and adenosine-5′-(β,γ-imido)triphosphate (AMPPNP) were obtained from Böhringer (Mannheim, Germany). The concentration of proteoliposomes was adjusted such that only one liposome was detected in the confocal volume at any time.

3. Results

EF_0F_1 was labelled with two fluorophores. In order to observe inter-subunit movement in functionally intact EF_0F_1 by intramolecular FRET, it is necessary to label specifically one subunit of the 'stator' part and one of the 'rotor' part. For labelling of the b-subunit with the FRET donor TMR, a cysteine in the b-subunit at position 64 was introduced. For labelling of the γ-subunit with the FRET acceptor Cy5, we used the mutant EF_1-γT106C [14]. Selective labelling was achieved by the following procedure.

Step 1: Isolated EF_0F_1-bQ64C was labelled with TMR at a substoichiometric concentration. A ratio of 0.2 TMR per EF_0F_1 was chosen to avoid labelling of both b-subunits. EF_0F_1-bQ64C-TMR was reconstituted into liposomes with approximately one EF_0F_1 per liposome. The rate of ATP synthesis was measured after energization by $\Delta pH/\Delta\phi$ with a rate of 61 s^{-1}. This rate is comparable to that of wild-type EF_0F_1 [22]. Therefore, labelling of the b-subunit with TMR did not change ATP synthesis activity (see Table 1). Step 2: F_1 parts of this enzymes were removed and liposomes with incorporated EF_0-bQ64C-TMR were retained. EF_0-liposomes showed no ATP synthesis activity, neither with TMR-labelled EF_0 nor with unlabelled EF_0, indicating an efficient removal of the F_1 parts. Step 3: EF_1-γT106C was isolated and labelled with Cy5. Step 4: Finally, we incubated the EF_0-liposomes with the EF_1-γT106C-Cy5 (and in control experiments with unlabelled EF_1-γT106C). After removal of the non-assembled F_1 parts by ultracentrifugation, we obtained EF_0F_1 in liposomes, in which the b-subunit in the EF_0 part was specifically labelled with TMR and the γ-subunit in the F_1 part was specifically labelled with Cy5. The ATP synthesis rate of this double-labelled EF_0F_1 was 33 s^{-1}, which corresponds to 50% of the rate before removal of F_1. A similar result was obtained when unlabelled EF_1 was reassembled with EF_0. Addition of DCCD blocked almost completely ATP synthesis (Table 1).

Using double-labelled enzymes reconstituted in liposomes ('FRET-labelled EF_0F_1', EF_0-bQ64C-TMR-F_1-γT106C-Cy5), intramolecular FRET between TMR at the b-subunit and Cy5 at the γ-subunit was measured by a confocal set-up with two-channel detection. Photons were detected in the spectral range of 545–610 nm from TMR ('green channel') and at wave-

Table 1
Rates of ATP synthesis catalyzed by labelled EF_0F_1 in liposomes

Conditions	Rate (s^{-1})
b-mutant	
EF_0F_1-bQ64C	61 ± 3
b-mutant after removal of F_1	
EF_0-bQ64C	0
labelled b-mutant	
EF_0-bQ64C-TMR-F_1	61 ± 4
labelled b-mutant after removal of F_1	
EF_0-bQ64C-TMR	0
labelled b-mutant after removal of F_1 and reassembled with F_1	
EF_0-bQ64C-TMR-F_1	29 ± 4
b-mutant after removal of F_1 and reassembled with labelled F_1	
EF_0-bQ64C-F_1-γT106C-Cy5	32 ± 6
labelled b-mutant after removal of F_1 and reassembled with labelled F_1	
EF_0-bQ64C-TMR-F_1-γT106C-Cy5	33 ± 3
EF_0-bQ64C-TMR-F_1-γT106C-Cy5 in presence of 10μDCCD	4 ± 3

EF_0F_1 with mutations bQ64C and γT106C was reconstituted into liposomes and the rates of ATP synthesis were measured after energization by ΔpH and $\Delta\phi$. Given are the mean and standard deviations from six independent measurements.

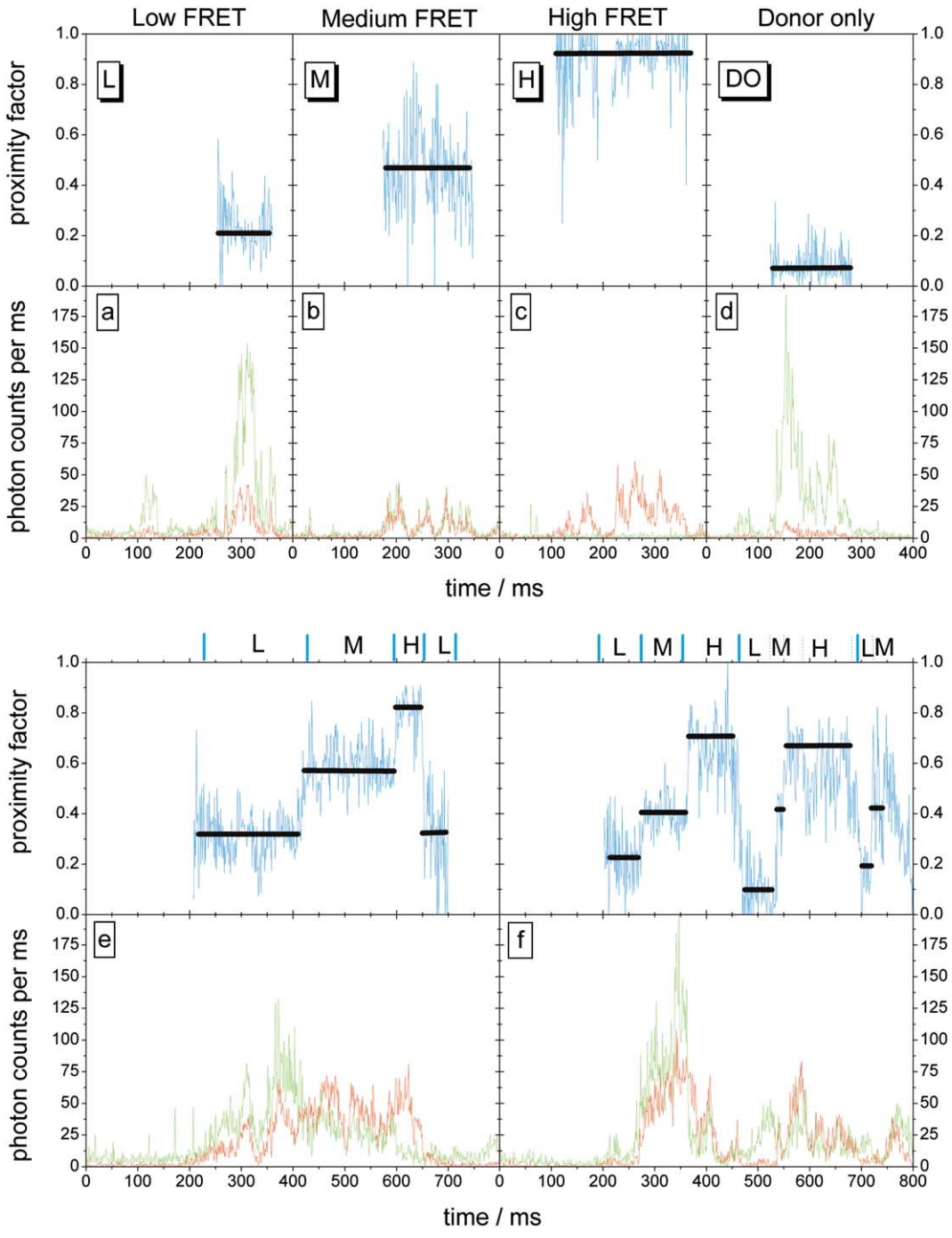

Fig. 1. Photon bursts and proximity factor traces of single FRET-labelled EF_0F_1 in liposomes. Single-molecule fluorescence of FRET-labelled EF_0F_1 reconstituted in liposomes is observed as photon bursts, when liposomes containing one FRET-labelled enzyme traverse the confocal detection volume. Green: time traces of TMR fluorescence intensities; red: simultaneous time trace of Cy5. The blue traces in the panels above are the calculated proximity factors. In the presence of AMPPNP, three states with different FRET efficiencies (a–c) are observed in which the proximity factor remains constant during the burst. d: Reconstituted EF_0-bQ64C-TMR-F_1 represent enzymes with FRET donor only. e,f: In the presence of ATP, fluctuations of the proximity factor corresponding to the three FRET states are detected within one burst. The sequence of states is indicated above the proximity factor trace.

lengths above 665 nm from Cy5 ('red channel'). The average background signal from buffer A containing 1 mM AMPPNP or 1 mM ATP was approximately 3 photons/ms in both channels. In the presence of FRET-labelled EF_0F_1-liposomes in solution, well-separated photon bursts with count rates up to 150 photons/ms were observed. This indicates a single liposome migrating through the detection volume. At the beginning of the time trace a in Fig. 1, both channels detect only background signal. At 255 ms, a liposome enters the detection volume and the TMR fluorescence intensity (green trace) increases to approximately 150 counts/ms. At 370 ms, the proteoliposome leaves the detection volume and and fluorescence decreases to background level. Simultaneously, an increase of Cy5 fluorescence with a count rate of 35 counts/ms is detected

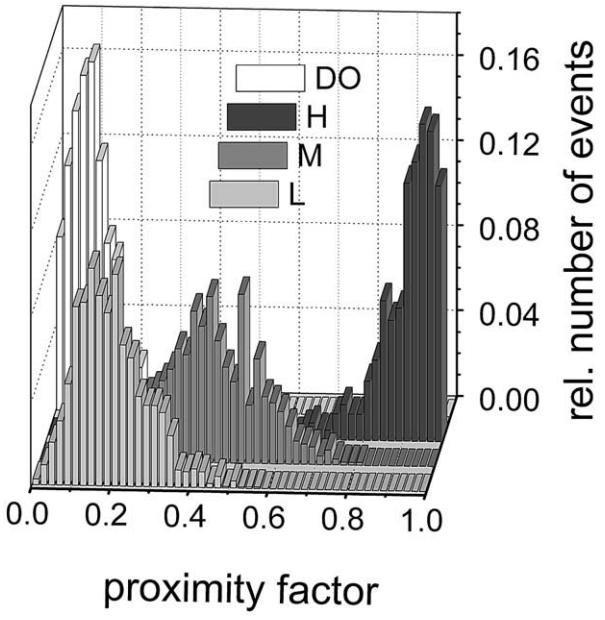

Fig. 2. Histogram of proximity factors from single FRET-labelled EF_0F_1 in the presence of 1 mM AMPPNP. L: low FRET efficiency state; M: medium FRET efficiency state; H: high FRET efficiency state; DO: 'donor only' state from TMR-labeled EF_0F_1 without FRET acceptor. The normalized distributions are calculated from 1 ms time intervals in 10 long-lasting photon bursts for each FRET state.

(red trace). Since Cy5 is not excited directly at the laser wavelength and power applied, this indicates inter-subunit FRET from TMR to Cy5.

There are two reasons for the fluctuations in fluorescence intensities. First, the proteoliposome moves through the focus driven by Brownian motion. Excitation power and fluorescence that is detected depend on the position of the enzyme within the confocal volume. Second, the efficiency of FRET changes with inter-subunit movement. FRET efficiency was calculated as the proximity factor P [26]. This factor is the ratio of the acceptor fluorescence intensity (I_A) divided by the sum of donor and acceptor intensities:

$$P = I_A/(I_D + I_A) \quad (1)$$

The blue trace in Fig. 1a shows the proximity factor for the event. Although there were large changes in the fluorescence intensities in both channels within the photon burst, the proximity factor remained nearly constant around $P = 0.2$. In the following, this state is called L ('low FRET state').

We calculated the proximity factors for different photon bursts and classified them into four FRET states: L ('low') with $P \approx 0.2$, M ('medium') with $P \approx 0.5$, H ('high') with $P \approx 0.8$ and DO ('donor only') with $P < 0.1$. Examples for the different states are shown in Fig. 1a–d. In FRET state M, fluorescence intensities of donor and acceptor are almost equal; in FRET state H, acceptor count rate is higher than donor count rate. In state DO, the acceptor fluorescence is near background. As a rare event, we also observed photobleaching of the acceptor fluorophore within the burst (data not shown) indicated by a spontaneous decrease of the acceptor count rate to background level and accompanied by a simultaneous rise of the donor count rate. Photobleaching of Cy5 resulted in state DO. The same fluorescence intensity ratio for the DO state with $P < 0.1$ was measured in control experiments using reconstituted EF_0-bQ64C-TMR-F_1, which contained only the donor fluorophore on the b-subunit. The DO state was observed in the FRET-labelled ATP synthase preparation, because only 65% of EF_1 contained Cy5 and, as a consequence, 35% of the reconstituted EF_0F_1 were lacking the acceptor label. Detection of some photons in the acceptor channel ('cross talk') is therefore due to the spectral properties of TMR. This cross talk leads to an 'apparent proximity factor' which does not reflect FRET. The states L, M, and H were attributed to different conformations of the H^+-ATP synthase. These are characterized according to the Förster theory of FRET [27] by different distances between the donor at the b-subunit and the acceptor at the γ-subunit. Therefore, FRET state L reflects a long distance, M a medium distance and H a short distance.

When single-enzyme fluorescence was measured in the presence of 1 mM ATP, well-separated photon bursts were observed. However, the time course within one burst was quite different. Fig. 1e,f show two examples during ATP hydrolysis. In contrast to the bursts in the presence of AMPPNP, there was a change in the green-to-red intensity ratio, which can be seen more clearly using the calculated proximity factor (blue trace). The proximity factors fluctuated within one photon burst. Three levels of the proximity factor could be distinguished (L, M and H), which were comparable in magnitude to the three different states observed in the presence of AMPPNP. During ATP hydrolysis, a definite order of proximity factor transitions was observed: from the low FRET (L) to the medium FRET (M) to the high FRET (H) and then again to the low FRET (L) state. In a few cases, we observed within this sequence L→M→H→L also 'backward steps' (see Fig. 1f, the high FRET state starting at 560 ms seems to oscillate between H and M states before reaching the L state at 700 ms) and in some photon bursts, the sequence

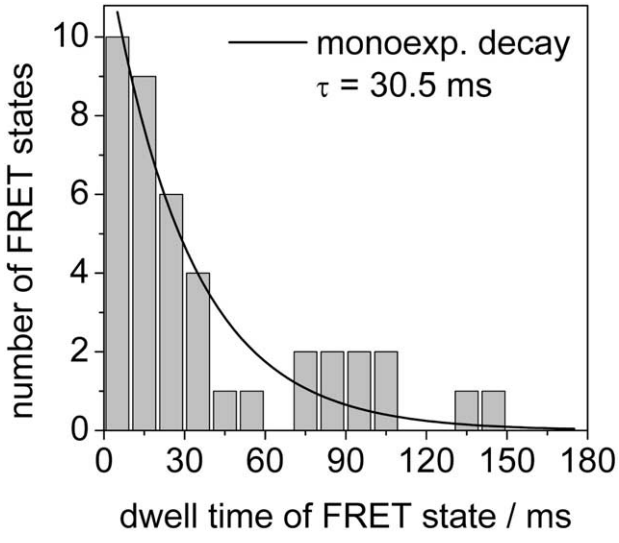

Fig. 3. Distribution of dwell times for the FRET states in presence of ATP. Dwell times of 40 FRET states were measured from photon bursts of FRET-labelled EF_0F_1 in liposomes, showing at least three distinct FRET states. First and last states of these bursts are omitted for the histogram, i.e. the states during entering and leaving the detection volume. The monoexponential decay fit yields a mean lifetime of 30 ms.

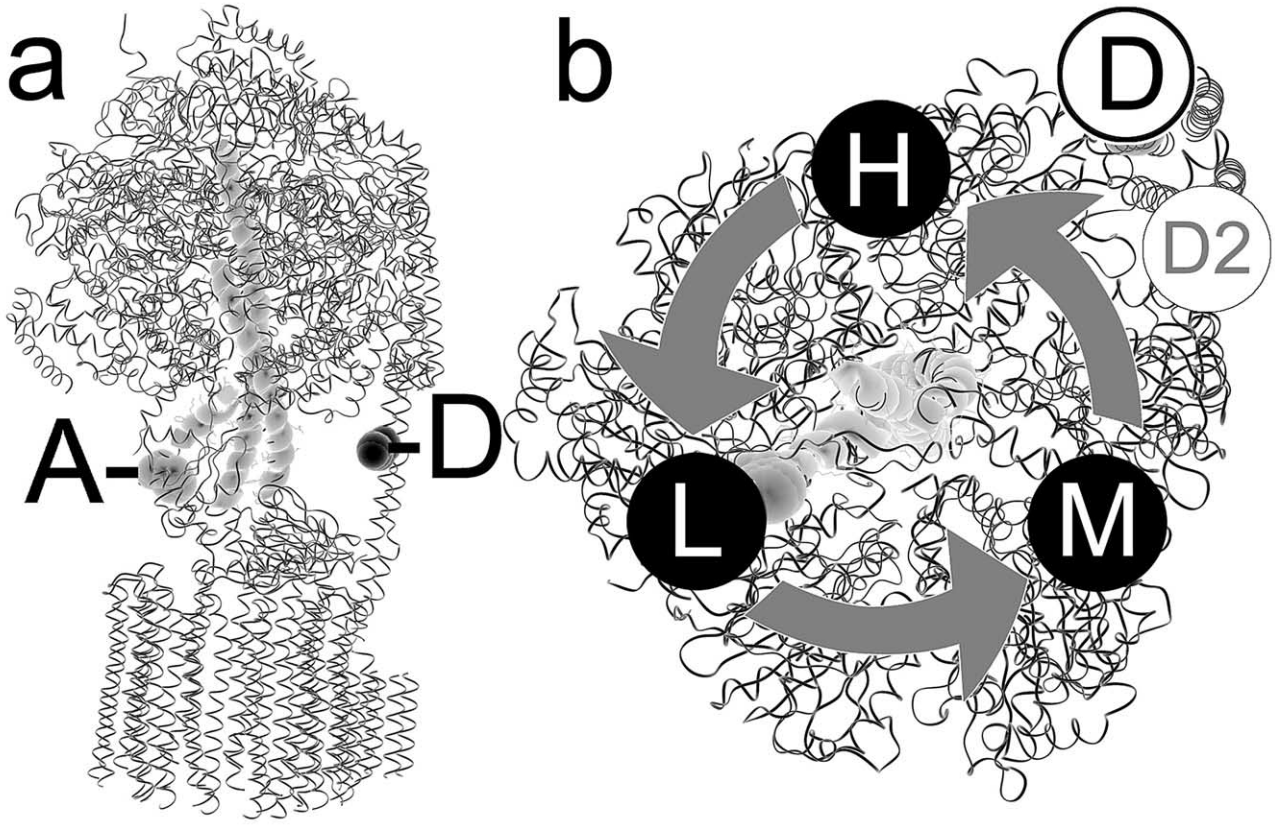

Fig. 4. Model of the FRET-label positions in EF_0F_1. Left: Side view of EF_0F_1. The homology model by S. Engelbrecht (http://131.173.26.96/se/se.html) was aligned with the structure of the γ- and ε-subunit [30]. Amino acid position of the FRET donor TMR at the b-subunit is indicated with 'D'; amino acid position of the FRET acceptor Cy5 at the γ-subunit is indicated with 'A'. Right: Cross section at the fluorophore level, viewed from the F_0 to the F_1 part. Position bQ64C is labelled 'D'; the distance between bQ64C and γT106C is labelled L (low FRET). Rotation of the γ-subunit by 120° steps results in two additional positions labelled M (medium FRET) and H (high FRET). The sequence L→M→H→L corresponds to counter-clockwise rotation of the γ-subunit during ATP hydrolysis.

was reversed (L→H→M→L). In all cases the movements occurred stepwise within our experimental time resolution for defining a FRET state (approximately 5 ms).

To calculate a mean value for the proximity factor, we chose 10 photon bursts for each FRET state from the experiments with AMPPNP, in which the sum of donor and acceptor intensities was more than 20 counts/ms. The number of events as a function of the proximity factors is plotted in Fig. 2. The mean values of these distributions are $P = (0.18 \pm 0.1)$ for L, $P = (0.47 \pm 0.1)$ for M and $P = (0.8 \pm 0.1)$ for H. For DO, an apparent value of $P = (0.1 \pm 0.05)$ was obtained.

From the experiments during ATP hydrolysis, we selected 40 long-lasting photon bursts and measured the duration of the different FRET states. These dwell times were sorted into 10 ms intervals. The distribution (Fig. 3) was fitted by a monoexponential decay function which yielded a lifetime of 30 ms. This means that the average lifetime of the FRET states is 30 ms. If one ATP molecule is hydrolyzed during one FRET state, this corresponds to a rate of ATP hydrolysis of 33 s^{-1}, which agrees with the rate measured under comparable conditions in bulk experiments [28].

4. Discussion

H^+-ATP synthase from *E. coli* carrying TMR at position 64 of the b-subunit and Cy5 at position 106 of the γ-subunit catalyzed ATP synthesis, when the proteoliposomes were energized ($\Delta pH \approx 4$ plus $\Delta\phi$). Thus, the attachment of the two fluorophores did not disturb the conformational dynamics of the enzyme during proton transport-coupled ATP synthesis. By a single-molecule detection method, we measured the fluorescence of both fluorophores simultaneously, when liposomes containing one FRET labelled enzyme traversed the confocal volume. The intramolecular FRET efficiencies were determined by calculating proximity factor P. Three different FRET states characterized by proximity factors of around 0.2, 0.5, and 0.8 were observed, corresponding to three different distances of Cy5 at the γ-subunit relative to TMR at the b-subunit. In the presence of AMPPNP, these distances remained constant within the photon bursts, i.e. the enzymes were 'arrested' in one of the three different states. In the presence of ATP, the same three states were observed as characterized by their proximity factors. However, during ATP hydrolysis fluctuations between these three states occurred within one burst. The average lifetime of each FRET state was 30 ms. Since the time resolution is limited by the integration time of 1 ms used in these experiments, we are only able to estimate the upper limit for the transition time between the different states, which is less than 3 ms.

During ATP hydrolysis the distance between the fluorophores at the γ-subunit and the b-subunit remains constant within one conformational state, until a fast transition to the next state occurs. In this new state, the enzyme remains again for a time interval followed by a fast transition to the third

state. From this third state, the next transition leads to the first conformation again. Therefore, the distances between the γ-subunit and the b-subunit change in a cyclic manner. Based on the current knowledge of the structure of F_0F_1, we interpret these data in a molecular model (Fig. 4) which is based on homology modelling and alignment of structural information. This is shown in a side view in Fig. 4a and as a cross section at the fluorophore level (viewed from F_0 to the F_1 part). With b-subunits located at the interface of one αβ-pair, the distance between bQ64C and γT106C is 7.3 nm in the model. When we start at this position (L) and rotate the γ-subunit in steps of 120° counter-clockwise around an axis going through the center of F_1, the distances between bQ64C and γT106C are approximately 5 nm (M) and 2 nm (H). These distances closely match the values calculated from the proximity factors, if we assume $R_0 = 5.3$ nm [29] according to the Förster theory of FRET [27].

In some photon bursts, we observed a clockwise order of transitions. We attribute these events to enzymes in which the second b-subunit is labelled with TMR (position 'D2' in Fig. 4b). However, in most experiments, the sequence of FRET states was L→M→H→L. This implies that the sequence of states observed in the single-molecule FRET experiments is in accordance with a counter-clockwise rotation of the γ-subunit relative to the b-subunit during ATP hydrolysis. We conclude from this result that the γ-subunit rotates stepwise relative to the b-subunit in fully active EF_0F_1 in liposomes.

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