Subunit movement in individual H\(^{+}\)-ATP synthases during ATP synthesis and hydrolysis revealed by fluorescence resonance energy transfer

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Abstract

F-type H\(^{+}\)-ATP synthases synthesize ATP from ADP and phosphate using the energy supplied by a transmembrane electrochemical potential difference of protons. Rotary subunit movements within the enzyme drive catalysis in either an ATP hydrolysis or an ATP synthesis direction respectively. To monitor these subunit movements and associated conformational changes in real time and with subnanometre resolution, a single-molecule FRET (fluorescence resonance energy transfer) approach has been developed using the double-labelled H\(^{+}\)-ATP synthase from *Escherichia coli*. After reconstitution into a liposome, this enzyme was able to catalyse ATP synthesis when the membrane was energized.

Subunit movements in F\(_{1}\) domains

The H\(^{+}\)-ATP synthase from *Escherichia coli* is a large membrane-bound enzyme that consists of eight different subunits. These subunits comprise two parts: the hydrophilic F\(_{1}\) part with subunit composition \(\alpha_3\beta_3\gamma\delta\varepsilon\) containing three catalytic nucleotide binding sites and the membrane-integrated F\(_{0}\) part with subunits \(\alpha_2\beta_2\gamma\delta\varepsilon\) containing the proton transport sites. The enzyme catalyses the synthesis of ATP using the electrochemical potential difference of protons across the lipid membrane and the reverse reaction, the proton transport-coupled ATP hydrolysis [1–3].

The \(\alpha\)- and \(\beta\)-subunits are arranged in a barrel-like hexameric structure around the central \(\gamma\)-subunit and catalysis is associated with a stepwise rotational motion of the \(\gamma\)- and \(\varepsilon\)-subunits relative to \(\alpha_3\beta_3\). First direct experimental evidence for a rotational mechanism of catalysis was provided by biochemical methods in 1995 using cross-links between the \(\gamma\)-subunit and the \(\beta\)-subunits specifically tagged with a radioactive label [4]. Spectroscopic methods using the photo-selection with polarized laser light were followed by the single-molecule anisotropy measurements of the \(\gamma\)-subunit rotation using immobilized F\(_{1}\) domains [5,6].

The most elegant way of a direct demonstration of \(\gamma\)-subunit rotation was the videomicroscopic experiment by Noji et al. in 1997 [7]. The authors attached the \(\alpha_3\beta_3\gamma\) fragment from the bacterium PS3 to a glass surface through the N-terminal histidine tags at the three \(\beta\)-subunits and used a 3 \(\mu\)m fluorescent actin filament bound to the \(\gamma\)-subunit as a pointer of the actual \(\gamma\)-subunit orientation. Starting hydrolysis by the addition of ATP resulted in the counterclockwise rotation of the actin filament, which was observed for several minutes. This approach was later refined using smaller polymer beads or magnetic beads. The original finding of a three-stepped rotation of the \(\gamma\)-subunit has emerged into a more complex picture with at least one intermediary angle-resolved substep and the distinct stopping positions of the \(\gamma\)-subunit have been correlated with the binding events of ATP, the catalytic reaction of ATP hydrolysis and the sequential release of the products ADP and phosphate [8].

The single-molecule FRET (fluorescence resonance energy transfer) approach with F\(_{0}\)F\(_{1}\)

ATP synthesis requires that the enzyme with all subunits is embedded into a lipid bilayer and, additionally, a proton motive force has to be applied. This has been achieved by reconstitution of the holoenzyme F\(_{0}\)F\(_{1}\) into the lipid vesicles. The required pH difference \(\Delta\text{pH}\) and the electric potential difference \(\Delta\Phi\) are generated by a rapid injection of the proteoliposomes (equilibrated at low pH and low internal K\(^{+}\) concentration) into a buffer at high pH and high K\(^{+}\) concentration in the presence of the K\(^{+}\) carrier valinomycin. Depending on a variety of parameters, ATP synthesis can be measured up to several minutes before \(\Delta\text{pH}\) and \(\Delta\Phi\) are dissipated [9,10].

To monitor the intramolecular rotary motions of the \(\gamma\)-, \(\varepsilon\)- or \(\zeta\)-subunits upon ATP synthesis, the size for the markers of rotation described above had to be reduced significantly. Organic fluorophores have a diameter of approx. 1 nm. When they are covalently bound to cysteine residues at appropriate sites, they do not prevent the rotation of subunits \(\gamma\) or \(\varepsilon\), which have to pass through the space between the peripheral...
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Figure 1 | Model of the H⁺-ATP synthase from E. coli according to [14]

(A) The membrane-embedded F₀-part is located at the bottom, the F₁-part is at the top. The rotating subunits are shown in black, the static subunits are shown in grey. Two positions for fluorophore labelling have been introduced into ‘off-axis domains’ of the rotor, utilizing a cysteine residue either in the γ-subunit (γ₁₀₆, [11–16]) or in the ε-subunit (ε₅₆). On the stator specific labelling either of a lysine residue on the β-subunits (β₄, [13]) or of a cysteine residue at the dimeric b-subunits (b₆₄, [11,12,14,15,17]) was used for single-molecule FRET studies. The arrow indicates the direction of rotation during ATP synthesis. (B) The FRET-labelled H⁺-ATP synthase in a liposome is diffusing through the confocal volume of laser excitation and photon detection.

stalk consisting of the b-subunit dimer and the central axis of rotation (Figure 1). We attach one fluorophore to an amino acid at the surface of either the γ- or ε-subunit and choose positions with large distances towards the expected axis of rotation in F₀,F₁ [11–17]. The radius of fluorophore rotation is estimated from the structure to be in the range of 3–3.5 nm. The rotation of γ or ε results in sequential distance changes between these off-axis parts of the subunits with respect to a fixed point outside the rotor. The shortest and the longest distances towards a second position at the fixed b-dimer are expected to be in the range 1–10 nm (i.e. the diameter of the F₁ part). The perfect spectroscopic method to detect distance changes in this range is FRET. FRET is the non-radiative process of energy transfer between two molecules or fluorophores [18]. The directly excited fluorophore is called the FRET donor, the second one is called the FRET acceptor and it is in the electronic ground state. Energy transfer from the donor to the acceptor results in a decrease of fluorescence intensity of the donor and an increase of fluorescence of the acceptor. The FRET efficiency depends on the distance between the two fluorophores, their spectral properties and the relative orientation of their transition dipole moments. Using rhodamines like TMR (tetramethylrhodamine) as the FRET donor and indodicarbocyanines like Cy5 as the FRET acceptor yields a 50% FRET efficiency at a distance of $R_0 = 6–7$ nm (‘Förster radius’) and, accordingly, reliable distance measurements can be made between 3 and 9 nm.

To carry out FRET measurements at the single-molecule level, the exciting light must be confined to a very small volume (a few femtolitres) to excite only one fluorophore [19]. This is achieved by diffraction-limited focusing of a laser beam with a microscope objective into the sample buffer. The sample contains a very low concentration of the labelled enzyme (100 pM), i.e. only 0.1 molecule is present in the focal volume on time average. When a proteoliposome reaches the laser focus during its diffusion (Figure 1B) the FRET donor fluorophore is frequently excited ($10^6$ s⁻¹). It relaxes either by emitting a photon (donor fluorescence) or by transferring the energy to the FRET acceptor (acceptor fluorescence). Confocal detection with a pinhole in the emission pathway rejects the out-of-focus fluorescence. Efficient optical filters in combination with single photon sensitivity of the avalanche photodiodes enable the observation of double-labelled enzymes during their diffusion through the confocal volume. The fluorescence is spectrally separated into two wavelength regions for separate detection of donor and acceptor. The stochastic diffusion process results in strong fluctuations of both fluorescence intensities. However, the FRET efficiency, $E_{\text{FRET}}$, i.e. the corrected ratio
between the acceptor fluorescence and the sum of both acceptor and donor fluorescence, remains constant as long as the intramolecular distance does not change.

Stepwise γ-subunit rotation in opposite directions

After the introduction of a cysteine residue in the b-subunits at position 64, we labelled it with TMR-maleimide as the FRET donor. The Cys106 of the γ-subunit was labelled with Cy5-monomaleimide as the FRET acceptor [11,12]. In the presence of AMP-PNP (adenosine 5′-[β,γ-imido]-triphosphate), a non-hydrolysable ATP analogue, three distinct FRET efficiencies were observed. The FRET states were called L state (low FRET efficiency; small energy transfer due to a large donor–acceptor distance), M state (medium FRET efficiency; approx. 50% transfer efficiency) and H state (high FRET efficiency; large energy transfer due to a short donor–acceptor distance). These FRET efficiencies remained constant within a photon burst (~100 ms), indicating a fixed distance between the two labels and, therefore, the absence of γ-subunit movements in this time range. In the presence of 1 mM ATP, fluctuations of the FRET efficiencies were detected within the photon burst, indicating a stepwise rotary motion of the γ-subunit during ATP hydrolysis. The FRET efficiencies, i.e. the FRET levels, were comparable with those in the presence of AMP-PNP; however, their magnitude changed stepwise and sequentially. FoF1 contains two b-subunits, and therefore, the donor was bound at two different sites, which have different distances to the acceptor. Unfortunately, we found two sequences of FRET level transitions during ATP hydrolysis with similar probability, in the L-M-H-L order as well as in the H-M-L-H order. Thus a correlation of one sequence with a unidirectional γ-subunit rotation during ATP hydrolysis was not possible.

In the next experiment, we labelled one lysine in the β-subunit at position 4 (see Figure 1A) with a rhodamine as the FRET donor and the γ-subunit at the position 106 with a indodicarbocyanine as the FRET acceptor [13]. Both labels were attached to the F1 part, before F1 was reassembled with F0 in liposomes. During ATP hydrolysis, a preferred sequence of FRET level transitions could be attributed to the counterclockwise rotation of γ. Changing the conditions to the ATP synthesis direction by generating ΔpH and ΔΦ in the presence of ADP and phosphate resulted in stepwise FRET-level transitions in the opposite direction. However, the signal-to-noise ratio had to be improved significantly.

Finally, we resolved the ambiguity of the localization on the b-subunits as follows: the donor TMR was attached to position γ106 and the two b-subunits were cross-linked with the acceptor Cy5-bismaleimide at the b64-position (Figure 1A) [14,15]. With this system we found the FRET level sequence H → M → L → H → during ATP hydrolysis for 72% of the bursts (see Figure 2A) and the reversed sequence L → M → H → L → during ATP synthesis for 83% of the burst (see Figure 2B). Independently, the FRET levels and the order of transitions were analysed by measuring the fluorescence lifetime of the FRET donor in the photon burst yielding the same result as the analysis of the fluorescence intensities.

Single-molecule dwell times and ensemble turnover rates

During catalysis, several FRET levels were found within each photon burst. The FRET level duration (‘dwell time’) was measured and summarized in histograms. The first and the last FRET levels have been omitted in the dwell time histograms since their duration (before entering and after leaving the observation volume) remain unknown. Therefore only the intermediary FRET levels of photon bursts with three and
more FRET levels contribute to the dwell time histograms shown in Figure 3. Depending on the time resolution of the fluorescence intensity trajectories (currently 1 ms, see Figure 2) we define a FRET level by a minimum duration of 4 ms and a maximum S.D. = 0.15 of the mean FRET efficiency of this level. Shorter dwell times are not taken into account and this is one reason for the many apparently ‘wrong’ FRET level sequences found during catalysis.

What are the biochemical events leading to the stepped rotation of the γ-subunit? Upon ATP hydrolysis at low ATP concentrations the video microscopic experiments with single F₁ fragments revealed an 80–90° rotation of γ associated with the binding of ATP to an empty catalytic site and a 40–30° rotation associated with the release of ADP and phosphate [20]. In our single-molecule FRET approach, we used 1 mM ATP, and therefore, the waiting time of the γ-subunit, i.e. the duration of a constant FRET level (‘dwell time’), is due to the hydrolysis reaction itself but not due to the delay until the next ATP molecule is bound to the enzyme. The mean dwell time τ_{hyd} was calculated from a monoexponential decay fit of the data in Figure 3(A) and yielded τ_{hyd} = 19 ms. The rate of ATP hydrolysis was calculated from biochemical ensemble measurements to yield ν_{hyd} = 67 s⁻¹, giving a turnover time of τ_{hyd} = 15 ms. Both rates are the same within error limits. Actually, we expected the single-molecule rate to be higher, since in this case only the active enzymes are contributing to the rate, whereas in the ensemble measurements the rate refers to all enzymes, i.e. the active and the inactive ones.

For ATP synthesis, this direct comparison between the single-molecule dwell times and the ensemble turnover is more complicated. The ensemble rates are determined from the initial turnover immediately after generating the ΔpH and Δψ. It includes all enzymes, active and inactive ones, and therefore, this rate should be smaller than the single-molecule rate as discussed for ATP hydrolysis. On the other hand, the single-molecule dwell times are collected between 30 s and 2 min after mixing. This time window was chosen since the data can be collected only after the disappearance of the turbidity changes due to mixing. However, dissipation of ΔpH and Δψ does not allow longer observation times. Therefore the actual value of the proton motive force experienced by the individual F₁Fₐ under observation is unknown and thus the measured dwell times cannot be correlated with the driving force for ATP synthesis. Despite these problems the single-molecule ATP synthesis turnover (τₛ = 51 ms) is in reasonable agreement with the ensemble turnover time (τ = 43 ms).

Some limitations of the single-molecule FRET approach with freely diffusing liposomes have to be overcome in future: (1) The observation time of a few hundred milliseconds is too short to allow monitoring of several repeating rotations. Possibly, this problem might be solved by immobilization of the proteoliposomes [21,22]. (2) The small number of detected photons per time interval limits the precision of the distance measurements. (3) Quenching of the protein-bound fluorophores like TMR [23] has to be avoided by the use of novel fluorophores with higher quantum yield and better photostability. This would allow further studies which give a detailed view of structural dynamics and conformational changes of a single H⁺-ATP synthase during catalysis.

We thank M. Diez, B. Zimmermann, S. Steigmiller and J. Petersen (Freiburg) and N. Zarrabi and M. Duser (Stuttgart) for their exceptional assistance. Parts of this work have been supported by the Landesstiftung Baden-Württemberg in the network of competence ‘Functional Nanodevices’.

References

Received 13 April 2005