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BIOPHYSICS LETTER

Fluorescence resonance energy transfer in single enzyme molecules with a quantum dot as donor

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Abstract H^+-ATPsynthases couple a transmembrane proton transport with ATP synthesis and ATP hydrolysis. Previously, the relative subunit movement during this process has been measured by fluorescence resonance energy transfer (FRET) between two organic fluorophores covalently bound to different subunits. To improve the photophysical stability, a luminescent CdSe/ZnS nanocrystal (quantum dot) was bound to the enzyme and an organic fluorophore, Alexa568, was used as fluorescence acceptor. Single-molecule spectroscopy with the membrane integrated labeled H^+-ATPsynthase was carried out. Single-pair FRET indicates three different conformations of the enzyme. During ATP hydrolysis relative intramolecular subunit movements are observed in real time.

Keywords Single pair FRET · Fluorescence resonance energy transfer · Nanomotor · Quantumdots · H^+-ATPsynthase

Introduction

Membrane bound H^+-ATPsynthases synthesize ATP from ADP and inorganic phosphate in bacteria, chloroplasts and mitochondria. This enzyme has attracted considerable attention in recent years, not only because of its importance for the bioenergetics of cells, but also because of the remarkable mechanism by which it makes ATP (Abrahams et al. 1994; Boyer 1998; Capaldi and Aggeler 2002; Junge et al. 1997; Kinosita et al 2004; Noji et al. 1997; Weber and Senior 2003). It consists of a hydrophilic F_1 part with subunits $\alpha_3\beta_3\gamma\delta\varepsilon$ and a hydrophobic membrane integrated F_0 part with subunits ab_2c_{10}. The H^+-ATPsynthase couples a transmembrane proton transport through the F_0 part with ATP synthesis and hydrolysis in the F_1 part. The coupling is effected by rotation of the rotor (subunits $\gamma\varepsilon c_{10}$) relative to the stator subunits $\alpha_3\beta_3\delta ab_2$. Single-pair FRET was used to measure the relative subunit movements in membrane-integrated F_0F_1 during catalysis. Previously, an organic fluorescence donor and an acceptor were bound covalently to specific sites (genetically introduced cysteines), one at the rotor subunit γ or ε and the other at the stator subunit b (Diez et al. 2004; Zimmermann et al. 2005, 2006). However, fluorophores with higher photostability and higher brightness are required for a more detailed analysis of the kinetics of the subunit movements with single-pair FRET. Especially, it is important to increase the observation time and the signal to noise ratio. Luminescent semiconductor nanocrystals (quantum dots, QD) have unique photophysical properties: high photostability, brightness and narrow size-tuneable fluorescence spectra (Alivisatos 1996; Bawendi et al. 1990; Murray et al. 1993). Quantum dots with hydrophilic shells have been used to construct a variety of QD-bioconjugates (for review see Bruchez et al. 1998, Michalet et al. 2005). However, there are only few reports on investigations of QD-labeled biomolecules using the ultra high sensitivity of single-pair FRET (Hohng and Ha 2004, 2005). One problem is the strong blinking effect, i.e., an intermittence of fluorescence emission, which impairs analysis of FRET measurements. The second problem is the size of QDs. Water-soluble QDs have

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hydrophilic shells with diameters of 20–40 nm, i.e., they are bigger than the biomolecules which are to be analysed and this might impair the function of the bound biomolecule. In addition, it has been reported that FRET with single QDs can only be observed when several fluorescence acceptors (Cy3 labeled maltose binding proteins) are bound to one QD (Clapp et al. 2003).

In this work we labeled the H^+-ATPsynthase with a hydrophilic QD and an organic fluorescence acceptor and we investigated ensemble FRET and single-pair FRET.

Materials and methods

The F_1-part of ATPsynthase of *E. coli* with the mutation εH56C and F_0F_1 containing the mutation bQ64C were prepared as described (Börsch et al. 2002). F_1-εH56C was labeled with Alexa568-maleimide same as that described by Zimmermann et al. 2005 for the labeling of F_1 which tetramethylrhodamine. The molar ratio of bound fluorophore to protein was calculated from the absorption spectrum using $\varepsilon_{579} = 92{,}000\ M^{-1}\ cm^{-1}$ and $\varepsilon_{280} = 40{,}100\ M^{-1}\ cm^{-1}$ for Alexa568 and $\varepsilon_{280} = 197{,}000\ M^{-1}\ cm^{-1}$ for F_1. The absorption coefficient at 280 nm was calculated from the amino acid composition of F_1 according to Gill and von Hipppel (1989). To bind QD490, the amino groups of the quantum dot (4–6 amino groups per QD490, Evident Technologies) were derivatized by the reaction with N-[β-maleimidopropyloxy]succinimide ester (BMPS, Pierce). QD490 (10 µM) in 100 mM MOPS/NaOH, pH 7.9, 200 µM $MgCl_2$ and 0.1% n-dodecylmaltoside (DDM, Glycon) was incubated with 0.1 mM BMPS for 120 min at room temperature. Unreacted BMPS was removed by a centrifugation column (Sephadex G-25 fine, Amersham Biosciences, pre-equilibrated with the same buffer). The b-subunit of F_0-bQ64C-F_1 (20 µM) was labeled with QD490-maleimide using a molar ratio of 1:1 in 100 mM MOPS/NaOH, pH 7.5, 200 µM $MgCl_2$ and 0.1% DDM at 0°C for 240 min. The labeling efficiency was determined from the absorption spectra using $\varepsilon_{470} = 40{,}000\ M^{-1}\ cm^{-1}$ and $\varepsilon_{280} = 422{,}000\ M^{-1}\ cm^{-1}$ for QD490 (Evident Technologies) and $\varepsilon_{280} = 340{,}000\ M^{-1}\ cm^{-1}$ for F_0F_1. F_0F_1-QD490 was incorporated into liposomes, the F_1 part was removed and the liposomes with the membrane-integrated QD490-labeled F_0 part were reassembled with F_1-εH56C-Alexa568, giving proteoliposomes with double-labeled F_0F_1. The enzyme activity (rate of ATPsynthesis) was measured as described by Zimmermann et al. (2005). Fluorescence spectra were measured with a Perkin Elmer LS 45 fluorometer. Single-molecule FRET experiments were carried out with a two channel confocal microscope (Börsch et al. 2002). Fluorescence of QD490 was excited at 476 nm with a Krypton ion laser.

Results and discussion

The water soluble CdSe/ZnS quantum dot, QD490 (T2 Evi Tag, Lake Placid Blue, Evident Technologies) carrying amino functional groups at the surface was derivatized with *N*-[β-maleimidopropyloxy]succinimide ester yielding QD490-maleimide. QD490-maleimide was then bound to F_0F_1 at the cysteine bQ64C, giving a F_0F_1–QD490 carrying one covalently bound quantum dot at the amino acid C64 of the stator subunit b. F_0F_1–QD490 and for comparision the unlabeled F_0F_1 were incorporated into liposomes and the rate of ATPsynthesis was measured. It resulted in an activity of $(20 \pm 5)\ s^{-1}$ for F_0F_1 and $(15 \pm 5)\ s^{-1}$ for F_0F_1–QD490, i.e., labeling with QD490 at the b-subunit does slightly decrease the enzyme activity. In the next step the F_1–part was removed and the F_1-depleted proteoliposomes were incubated with F_1–Alexa568. This finally gives the membrane integrated double labeled H^+-ATPsynthase F_0F_1QD490–Alexa568 (see "Materials and methods").

The absorption spectra and the normalized fluorescence spectra of QD490 and Alexa568 are depicted in Fig. 1 showing the overlap between emission of QD490 and absorption of Alexa568. For a quantitative analysis the labeling degree of F_0F_1 with QD490 was determined spectroscopically. The absorption spectra of F_0F_1QD490 and QD490 were measured and the difference spectrum, i.e., the spectrum of F_0F_1, was calculated. From the difference absorbance at 280 nm the concentration of F_0F_1 was determined from the absorbance at 470 nm the concentration of QD490 was calculated:

$$\frac{c(QD)}{c(F_0F_1)} = \frac{A_{470}(QD)\varepsilon_{280}(F_0F_1)}{\varepsilon_{470}(QD)\Delta A_{280}(F_0F_1)} = 0.9 \quad (1)$$

The labeling degree of F_1–Alexa568 was determined (Zimmermann et al. 2005) similarly.

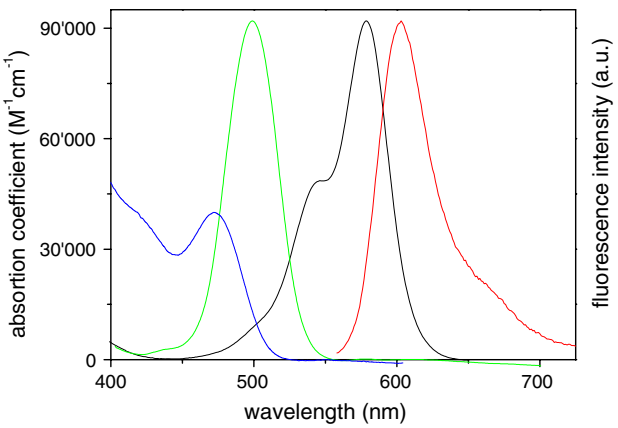

Fig. 1 Absorption coefficients of QD490 (*blue*) and Alexa568 (*black*) and normalized fluorescence spectra of QD490 (*green*) (excitation at 380 nm) and Alexa568 (*red*) (excitation at 550 nm)

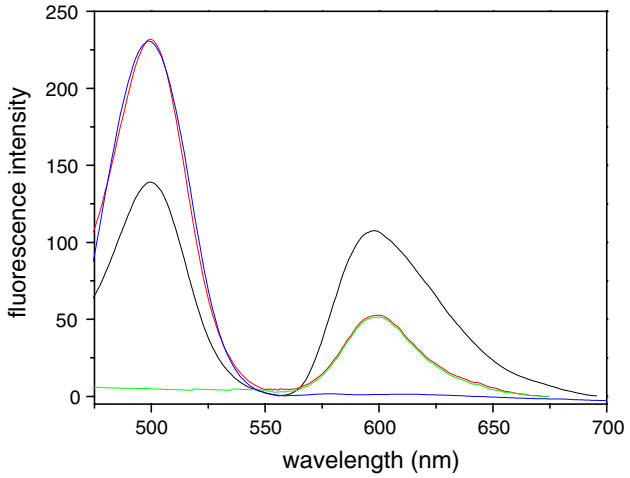

Fig. 2 Fluorescence emission spectrum of F0F1QD490–Alexa568 (*black*) and a mixture of QD490 (0.9 μM) and Alexa568 (0.5 μM) (*red*). For comparison the fluorescence spectra of the pure QD490 (*blue*) and pure Alexa568 (*green*) are shown. Measurements are carried out in 100 mM MOPS-NaOH, pH 7.5

The fluorescence emission spectrum of F_0F_1QD490–Alexa568 (excitation at 450 nm) is depicted in Fig. 2 showing peaks at 500 nm (emission of QD490) and at 600 nm (emission of Alexa568). The emission at 600 nm might be due to FRET; however also a direct excitation of Alexa568 might be possible. To investigate this, the concentrations of QD490 and of Alexa568 are calculated from the labeling degree and the fluorescence spectrum of a mixture of 0.9 μM QD490 and 0.5 μM Alexa568 is measured. A comparison of both spectra shows that fluorescence of F_0F_1QD490–Alexa568 at 500 nm is smaller, that at 600 nm is higher than in the mixture, i.e., there is FRET from QD490 to Alexa568. It is possible that FRET might occur also in the mixture of QD490 and Alexa568. Therefore, the spectra of both fluorophores are measured separately using the same concentrations as in the mixture. The fluorescence at 490 nm is the same in pure QD490 and in the mixture; similarly, the fluorescence at 600 nm is the same in pure Alexa568 and the mixture. This indicates that there is no FRET in the mixture, i.e., the distances between the fluorophores are too large for FRET. In F_0F_1QD490–Alexa568 both fluorophores are close enough to show FRET. It should be mentioned that all emission spectra are obtained with excitation at 450 nm. At this wavelength the absorbance of Alexa568 is very low, nevertheless, as can be seen in the fluorescence spectrum, there is some direct excitation of Alexa568.

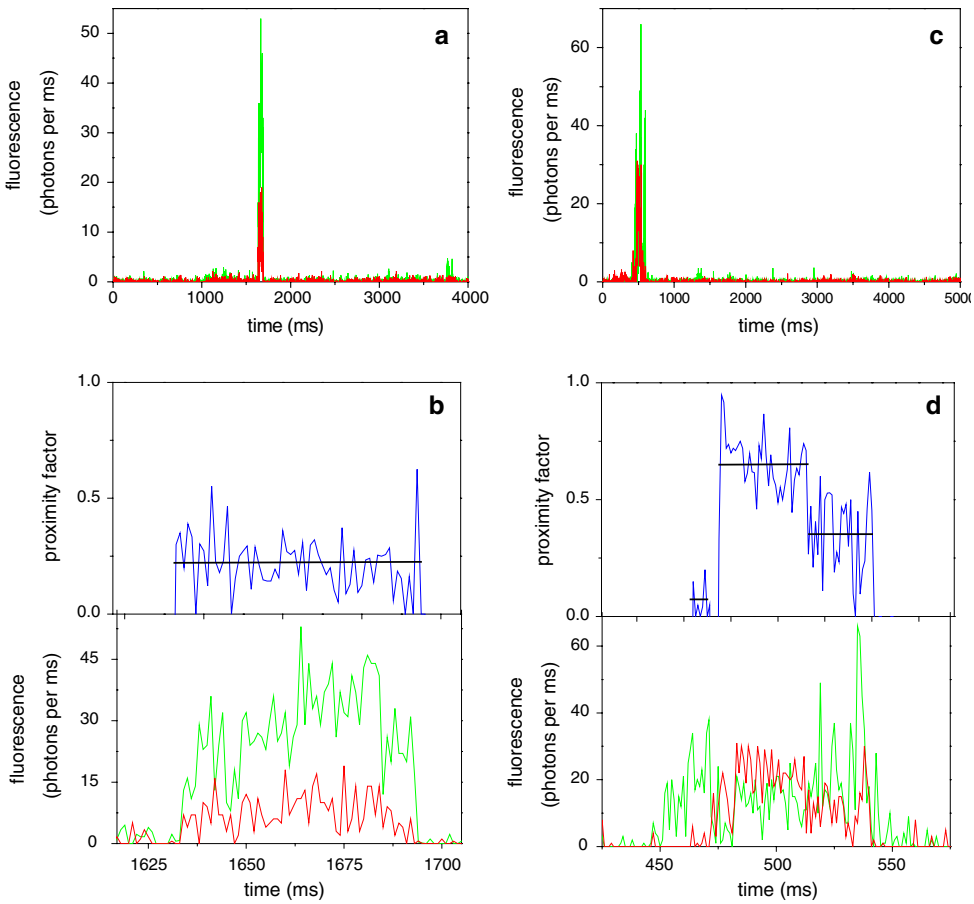

Fig. 3 Photon bursts from double labeled F0F1QD490–Alexa568 integrated in liposome membranes (excitation at 476 nm). Color code: Donor intensity, FD, QD490 (*green*), acceptor intensity, FA, Alexa568 (*red*); proximity factor *FA*/(*FA* + *FD*) (*blue*). The mean proximity factor is shown as *black line*. Proteoliposomes are suspended in buffer containing 100 mM MOPSNaOH, pH 7.5, 20 mM succinic acid, 2.5 mM MgCl2, 0.6 mM KCl. **a** photon burst in the absence of ATP, **b** photon burst from **a** with expanded time scale and calculated proximity factor, **c** photon burst in the presence of ATP, **d** photon burst from **c** with expanded time scale and calculated proximity factor

Based on these ensemble measurements, we try to observe FRET in a single-molecule experiment. FRET was measured with a confocal microscope, which allows detection of fluorescence photons from the donor and acceptor with single-molecule sensitivity (Diez et al. 2004). The laser was focussed in a droplet of buffer (80 μL) on a microscope slide to give a confocal volume of 7 fL. The labeled F_0F_1-liposomes (F_0F_1QD490–Alexa568) were diluted to approximately 70 pM and the diffusion of a proteoliposome through the confocal volume resulted in the detection of a large number of photons from the donor and acceptor during the transit time ("photon burst").

Figure 3a and c, show two examples of photon bursts in the absence of ATP (a) and in the presence of ATP (b) (donor fluorescence green, acceptor fluorescence red). When no proteoliposomes are in the confocal volume, only background noise is observed in the donor and acceptor channel. The proteoliposomes migrate statistically into and out of the confocal volume giving rise to photon bursts. Figure 3b and d show the photon bursts from a and c with an expanded time scale. The fluctuation of the fluorescence intensities result from the statistical movement of the proteoliposome, exposed to the intensity distribution of the exciting light within the confocal volume. From the fluorescence intensities in the donor (F_D) and acceptor (F_A) channel the proximity factor, $p = F_A/(F_A + F_D)$, was calculated (blue traces in Fig. 2b, d). The proximity factor describes the efficiency of energy transfer and only depends on the distance between donor and acceptor within the enzyme and especially, it does not depend on the intensity of exciting light.

In the absence of ATP, when the enzyme does not carry out catalysis, the proximity factor is constant, i.e., the distance is constant within a photon burst. Figure 2b shows a burst with the proximity factor 0.22. Analysis of 333 photon bursts in the absence of ATP resulted in three groups of photon bursts with low, medium and high proximity factors. Analysis at the data give the following average proximity factors with standard deviation: 0.18 ± 0.1, 0.48 ± 0.1 and 0.78 ± 0.1.

In the presence of ATP during ATP hydrolysis also three different proximity factors are observed. Analysis of 56 bursts resulted in the following average proximity factors with standard deviations: $p = 0.18 \pm 0.15$, 0.50 ± 0.15 and 0.75 ± 0.15. Within one burst, stepwise changes between the proximity factors are observed. The sequence of the change was low → high → medium → low in 80% of the analyzed bursts. This indicates a relative movement of subunits during catalysis. These data show that quantum dots can be used as energy donors in single-pair FRET to detect intramolecular conformational changes within the F_0F_1-nanomotor during catalysis.

In this work, we did not attempt to determine the exact distances between donor and acceptor. We showed that binding of a large quantum dot to a complex enzyme like the F_0F_1 nanomotor can be carried out without impairing enzyme activity and that FRET changes can be observed with a large QD as donor during catalysis. The high photostability and brightness of the QD will allow longer observation times of the same molecule and this is necessary for investigations of enzyme kinetics with single enzymes (Xie and Lu 1999).

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