Quantum dots for single-pair fluorescence resonance energy transfer in membrane-integrated EF_oF_1

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Abstract

spFRET (single-pair fluorescence resonance energy transfer) with organic fluorophores has been used to demonstrate rotation of the subunits γ and ε in membrane-integrated F_oF_1 during proton transport-coupled ATP synthesis. Owing to the high light intensities used in single-molecule spectroscopy, organic fluorophores show a high probability for photobleaching. Luminescent CdSe/ZnS nanocrystals with a hydrophilic shell have been covalently bound to F_oF_1 either to the stator subunit b or to the rotor subunit c. TIRFM (total internal reflection microscopy) shows that covalent binding of the QD (quantum dot) via cysteine to F_oF_1 leads to a significant decrease in the blinking probability in the microsecond-to-second time range. This effect allows the observation of subunit movements in an extended time range. If the QD is bound to the rotor subunit c, the fluorescence anisotropy shows fluctuations in the presence of ATP, in contrast with the constant anisotropy observed in the absence of ATP.

Rotation driven by ATP hydrolysis

F_oF_1-ATP synthases couple the transmembrane proton transport with synthesis of ATP from ADP and phosphate. They are membrane-bound enzyme complexes with a hydrophilic F_1-part (subunits $\alpha_3\beta_3\gamma\delta\varepsilon$) containing the nucleotide-binding sites, and a hydrophobic F_o-part (subunits ab_2c_{10}) involved in proton transport. Enzyme kinetic analysis showed a strong co-operativity of the three catalytic sites localized on the β-subunits. The binding change theory proposed three different conformations of the catalytic sites which are generated by different interactions of the γ-subunit with the β-subunits [1,2]. The X-ray structure of the F_1-part showed directly the three conformations of the β-subunit and their interactions with the γ-subunit [3]. The synchronization of the events on the three catalytic sites is effected by rotation of the γ-subunit within the $\alpha_3\beta_3$ barrel, i.e. the conformations of the three β-subunits are changed simultaneously during catalysis.

The rotational movement of the γ-subunit in the F_1-part was observed in different experiments: chemical cross-linking between γ- and β-subunits revealed that the γ-subunit interacts with different β-subunits during ATP hydrolysis [4]. Rotation of the eosin-labelled γ-subunit in immobilized F_1-parts was shown with polarized absorption recovery after photobleaching [5]. A fluorescence-labelled actin filament was attached to the γ-subunit at an immobilized F_1-part, and its rotation during ATP hydrolysis was observed by fluorescence microscopy [6,7].

A model for the coupling between proton translocation and rotation has been developed [8]. The ten c-subunits form a ring in the membrane and the protonation of aspartate (c61) occurs through the entrance channel from the side of high proton concentration. Deprotonation of c-subunits occurs through an exit channel to the side with low proton concentration. The protonation/deprotonation reactions on both sides together with the Brownian movements of the c-subunits lead to a rotation of the c-ring [8]. The γ- and β-subunits interact with the c-ring and, thereby, the rotational movement of γ and ε is coupled with the rotation of the c-ring. Rotation of the c-ring has also been observed with immobilized F_oF_1 during ATP hydrolysis by using a fluorescent actin filament as the indicator [9–12].

Rotation driven by proton transport

Movement of subunits during ATP synthesis has been shown by two different methods [13,14]. In order to investigate proton transport-driven rotation with FRET (fluorescence resonance energy transfer) in single enzyme molecules, we constructed an enzyme labelled with two fluorophores. According to the Förster theory, the efficiency of energy transfer depends strongly on the distance between the fluorophores [15]. The FRET efficiency, E_{FRET}, can be calculated from the fluorescence intensities of the donor (F_D) and acceptor (F_A):

$$E_{FRET} = \frac{F_A}{F_A + \gamma F_D} = \frac{R_0^6}{R_0^6 + r_{DA}^6}, \quad \gamma = \frac{\eta_A \phi_A}{\eta_D \phi_D} \qquad (1)$$

Key words: ATP synthesis, membrane-integrated F_oF_1, proton transport, quantum dot, rotation, single-pair fluorescence resonance energy transfer (spFRET).

Abbreviations used: FRET, fluorescence resonance energy transfer; NV, nitrogen-vacancy; QD, quantum dot; spFRET, single-pair FRET; TIRFM, total internal reflection microscopy; TMR, tetramethylrhodamine.

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with the Förster radius R_0; the distance between the donor and acceptor r_{DA}; the correction factor γ, with the detection efficiency of photons in the acceptor and donor channel η_A and η_D; and the quantum yield of the acceptor and donor Φ_A and Φ_D respectively.

We have built a confocal microscope which allows the time-resolved measurements of fluorescence photons from the donor and acceptor fluorophores with single-molecule sensitivity [16,17].

The donor/acceptor-labelled H^+-ATP synthase was constructed as follows. The peripheral stalk is part of the stator $\alpha_3\beta_3\delta ab_2$ of the enzyme. As the site for the acceptor fluorophore we chose that region in the peripheral stalk which is located between the membrane and the F_1 part. A cysteine residue was genetically introduced at b64. Since there are two b-subunits in F_oF_1, we used for labelling the fluorophore bisCy5, which reacts with the cysteine residues b64 in both b-subunits and forms a cross-link between them. Since the fluorophore is attached to the enzyme by two covalent bonds, it has a well-defined position.

The second fluorophore was attached to one of the rotor subunits. We used either the γ-subunit (mutant γT106C) or the ε-subunit (mutant εH56C) and labelled it covalently with the fluorescence donor TMR (tetramethylrhodamine)–maleimide. To obtain a selective double labelling of the enzyme, we used the following procedure [18,19]. F_oF_1 (mutant bQ64C) was isolated, labelled with bisCy5, reconstituted into liposomes and the F_1-part was removed. F_1 (mutant T106C or mutant εH56C) was isolated, labelled with TMR and bound to the F_1-depleted liposomes. Thereby, ATP synthesis activity was restored. The fluorophores were attached to a rotor subunit (γ or ε) in the region between the F_1-part and F_o-part. They are small so that, according to the current structural models of F_oF_1, no restriction of the rotational movement is expected. This system allows us to investigate relative subunit movements within the membrane-integrated F_oF_1 in freely diffusing proteoliposomes. The measurements of spFRET (single-pair FRET) gave the following results:

(i) The γ-subunit rotates within F_oF_1 in three steps during proton transport-coupled catalysis. The direction of rotation during ATP synthesis is opposite to that during ATP hydrolysis [18].

(ii) The ε-subunit rotates within F_oF_1 in three steps during proton transport-coupled catalysis. Again the direction of rotation during ATP synthesis is opposite to that during ATP hydrolysis [19].

(iii) In both cases the movement is a stepped rotation. The docking time at a given $\alpha\beta$-pair (dwell time) is between 10 and 50 ms and this time is related to the rate of catalysis. The switching time between two subsequent docking positions is lower than 0.2 ms [20]

QDs (quantum dots) bound to F_oF_1

In single-molecule spectroscopy, the intensity of the laser beam is very high in the confocal volume [20], resulting in a rapid photobleaching of the organic fluorophores. This problem might be solved using luminescent nanocrystals (QDs) as fluorescence donors. QDs have unique photophysical properties: high photostability, brightness and narrow size-tuneable emission spectra [21–23]. For applications in aqueous systems, QDs with hydrophilic shells have been synthesized and a variety of QDs with different hydrophilic shells and functional groups are commercially available. Different QD-bioconjugates have been constructed and they have found wide application in biochemistry and biology [24–26]. However, applications of QDs as donors in spFRET are rare. The main problem is the intermittence of fluorescence emission ('blinking'), observed with single QDs [27–29], which complicates the interpretation of FRET data. Previously, it has been reported that addition of thiols to the buffer containing the water-soluble QDs drastically reduces blinking, and spFRET with QD-labelled DNA was measured [30,31].

In order to improve the spFRET-F_oF_1 system, we investigated the possibility of using a QD as fluorescence donor. QD490 (Lake Placid Blue) supplied by Evident Technologies was used for our measurements. QD490 is a CdSe/ZnS QD surrounded by a hydrophilic poly(ethylene glycol) shell (diameter 11 nm) containing functional amino groups. In order to bind QD490 covalently to F_oF_1, the amino groups were first derivatized with *N*-[β-maleimidopropyloxy]succinimide ester giving QD490-maleimide. Under physiological conditions, QD490-maleimide reacts with the thiol group of a cysteine residue in the protein, forming a covalent bond. QD490 was bound to F_oF_1 at the cysteine b64C, giving a fluorescent labelled F_oF_1 with one covalently bound QD at the stator subunit b of the peripheral stalk.

The intermittence of fluorescence (blinking) is a severe problem for the use of QD in spFRET. Presumably, blinking is caused by surface defects, which might be modified by reaction with thiols or other reagents [30–32]. We investigated whether binding of QD490 to F_oF_1 changes the blinking behaviour. The blinking behaviour in the microsecond-to-second time range is of particular interest, since in this time range changes of spFRET in F_oF_1 are expected.

QD490 and F_oF_1-QD490 were immobilized on a microscope coverslip by adsorption at the glass surface. They were illuminated by evanescent field excitation through the edge of the microscope objective with a krypton ion laser at 476 nm by using TIRFM (total internal reflection microscopy). Images of QD490 (Figure 1A) and F_oF_1-QD490 (Figure 1B) were taken with a CCD camera (charge-coupled-device camera). Each luminescent spot represents one QD. Examples of the fluorescence time traces of one QD (marked with a circle) are shown in Figures 1(C) and 1(D). This 'on–off' reaction indicates that each luminescent spot is due to the emission of a single QD. The highest fluorescence intensity is approx. 40 photons/ms; the background intensity is approx. 30% of the maximal intensity. Both QD490 and F_oF_1-QD490 show almost the same maximal intensity; however, in F_oF_1-QD490, the blinking probability is drastically reduced in the microsecond-to-second time range. This result opens the possibility of using QDs in spFRET as fluorescence donors, if they are attached to F_oF_1 via a cysteine residue.

Figure 1 | Images and fluorescence time traces of single immobilized QDs and QDs bound to F_oF_1-ATP synthases

(**A**, **B**) TIRFM images of the water-soluble QD QD490 (**A**) and F_oF_1-QD490 (**B**), both adsorbed at a glass surface. QD490 is bound correctly at the b-subunit. Fluorescence intensity (number of photons per 50 ms) time traces are shown for the encircled QD490 (**C**) and the encircled F_oF_1-QD490 (**D**).

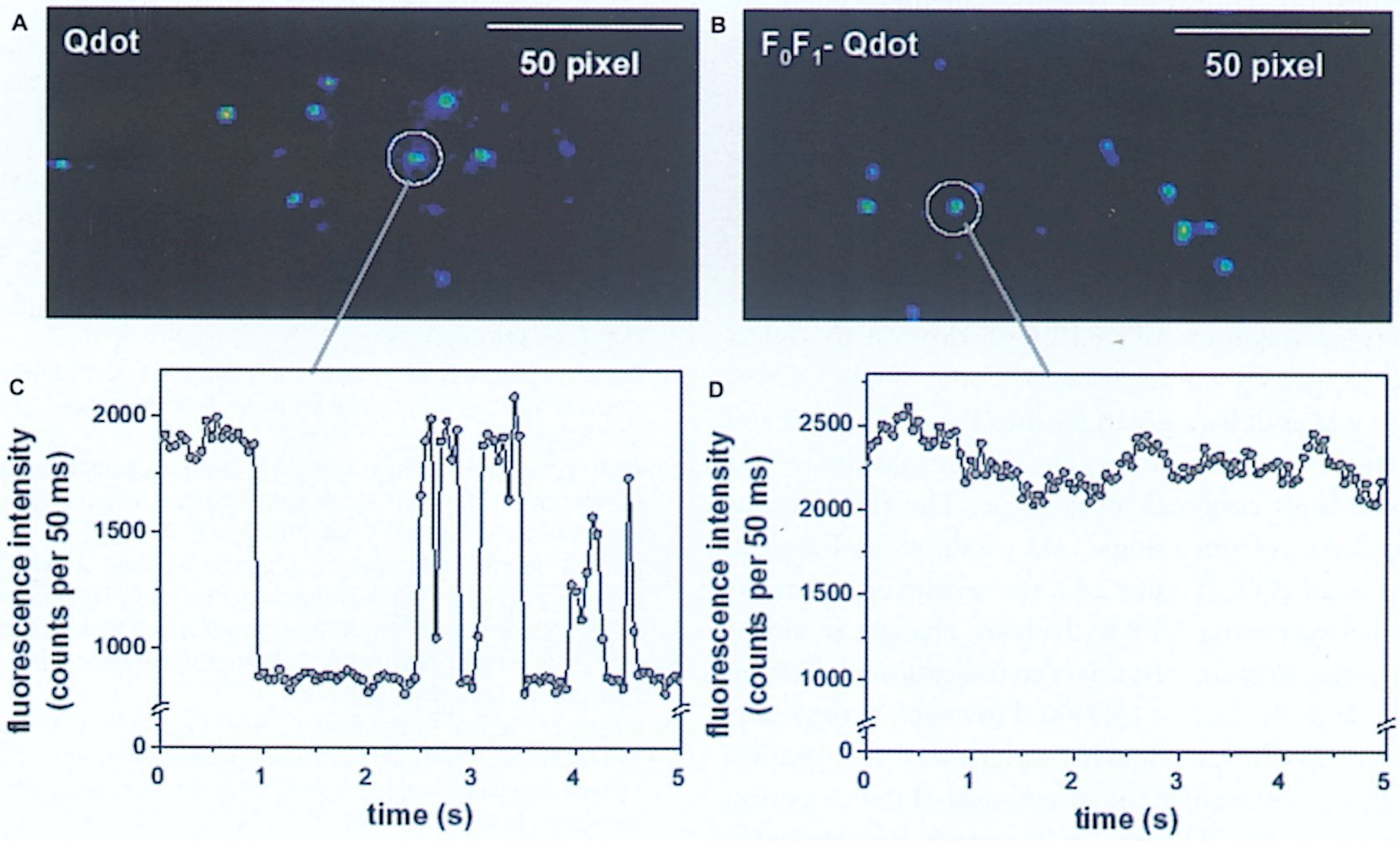
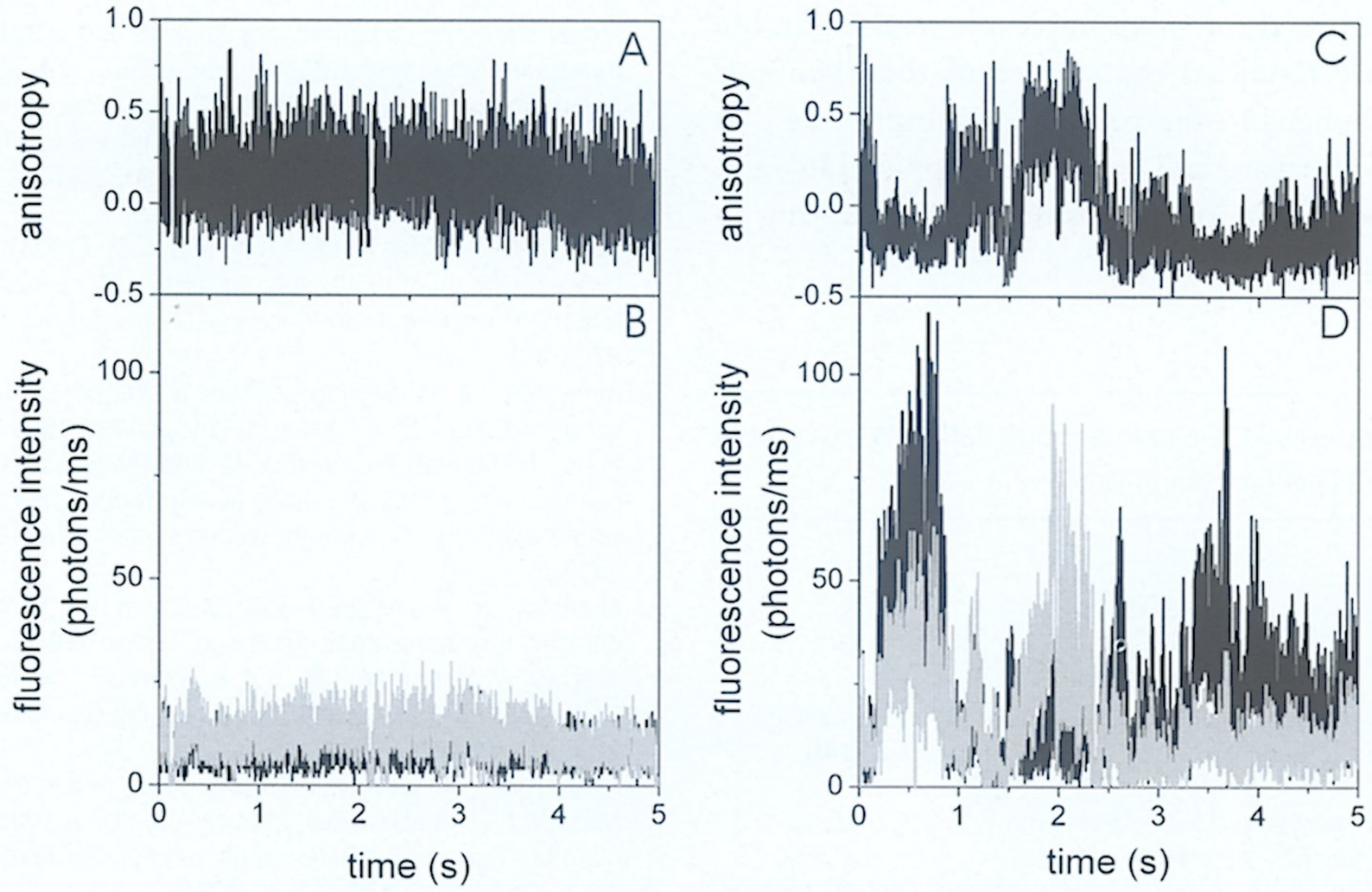


Figure 2 | Intensity time traces of polarized fluorescence emission of F_oF_1-QD490 adsorbed at a glass surface

QD490 is bound at the c-subunit ring of F_oF_1. Fluorescence with orthogonal orientation of the polarizers is shown in black and parallel orientation in grey. (**A** and **B**) Without ATP; (**C** and **D**) in the presence of 1 mM ATP. The calculated anisotropy is shown above the fluorescence traces.



The large size of QDs with hydrophilic shells is a disadvantage for measuring small distance changes from the donor to an acceptor. According to the Förster theory, the highest sensitivity to detect small movements is near the Förster radius. Its magnitude depends on the properties of the donor and acceptor, and therefore appropriate pairs have to be selected for each measurement to obtain optimal signals in the corresponding range of distances.

A further possibility of investigating rotational movements in F_oF_1 is based on the anisotropy of fluorescence emission of

a QD. It is possible to detect subunit rotation by measuring time-dependent changes in the anisotropy of a bound QD. This method has the advantage that only one fluorophore must be covalently bound to the enzyme, which facilitates the biochemical procedures for selective labelling. There are two disadvantages: the enzyme must be immobilized on a surface and the QD must be attached to a rotor subunit.

To show the feasibility of this approach, we used a mutant that carries a cysteine residue in the c-subunit (c2C). QD490 was bound covalently to these cysteine residues as described above for binding at the b-subunit. The c2C position is located outside of the membrane and opposite to the F_1-part. The labelled enzyme was immobilized by adsorption to a glass surface and imaged by confocal microscopy. Single QD490 fluorescence was split by a polarizer into the orthogonal and parallel polarization components. The time resolution is 1 ms using a home-built confocal microscope. The fluorescence intensity time traces from a single QD are shown in Figure 2. In the absence of ATP (Figure 2A), the anisotropy remains constant, whereas during ATP hydrolysis, changes in anisotropy can be seen. Presumably, this is an indication of rotation of the c-ring with the bound QD490. However, using fluorescence anisotropy for an angular resolution of the rotation only allows us to distinguish the orientation of the transition dipole moment of the QD between 0° and 90° for symmetry reasons.

A promising new sort of fluorescent nanocrystal is the NV (nitrogen-vacancy) colour centre in diamonds. NV centres are absolutely photostable with a quantum yield of nearly 100 % and show no blinking behaviour [33,34]. Fluorescent nanodiamonds with a diameter smaller than 20 nm have been produced and used for single-molecule tracking in life cells [35]. We have modified the surface of the diamonds for covalent attachment to the rotating γ-subunit in F_1 to monitor stochastic forward and backward stepping [36], and apply these diamonds for rotation studies of the c-ring in F_oF_1 in ongoing experiments.

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