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Conformational dynamics of the rotary subunit F in the A\(_3\)B\(_3\)DF-complex of Methanosarcina mazei Gö1 A-ATP synthase monitored by single-molecule FRET

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Abbreviations: CTD, C-terminal domain; NTD, N-terminal domain; smFRET, single-molecule Förster resonance energy transfer;
Abstract

In archaean the A₁A₀ ATP synthase uses a transmembrane electrochemical potential to generate ATP, while the soluble A₁ domain (subunits A₃B₃DF) alone can hydrolyse ATP only. The three nucleotide-binding AB-pairs form a barrel-like structure with a central orifice that hosts the rotating central stalk subunits D and F. ATP binding, -hydrolysis and product release cause a conformational change inside the A:B-interface, which enforces the rotation of subunits D and F. Recently, we reported that subunit F is a stimulator of ATPase activity. Here, we investigated nucleotide-dependent conformational changes of subunit F relative to subunit D during ATP hydrolysis in the A₃B₃DF-complex of the Methanosarcina mazei Gö1 A-ATP synthase using single-molecule Förster resonance energy transfer (smFRET).
1. Introduction

Archaea-type ATP synthases (A₁A₂O ATP synthases) are membrane proteins that use a transmembrane electrochemical potential of H⁺ or Na⁺ to generate ATP from ADP and Pᵢ [1]. In the reverse reaction they hydrolyse ATP to pump ions across the membrane. A₁A₂O ATP synthases are composed of nine subunits in a proposed stoichiometry of A₃:B₃:C:D:E₂:F:G₂:a:cₓ (Fig. 1). Ions are conducted by the membrane-embedded A₂O portion, while the chemical reaction is located in the soluble A₁ domain. The two domains are connected via the central stalk subunits D, F and C, and by two peripheral stalks consisting of heterodimers of subunits E and G. Ion pumping through the ring of c subunits is coupled to the catalytic centres in A₃B₃ via the central stalk subunits D, F and C. Most recently, ATPase-dependent power strokes of the A₃B₃DF-complex of the *Methanosarcina mazei* Gö1 A-ATP synthase (*MmA₃B₃DF*) were demonstrated, occurring in 120° steps at saturating MgATP concentrations [2]. The catalytic nucleotide-binding sites are located in the interface of each AB pair, with the main part inside the catalytic A subunit. During ATP hydrolysis each nucleotide-binding site is binding ATP, hydrolysing ATP to ADP and Pᵢ, and releasing the products in an alternating sequence that determines the conformation of the three AB pairs. Their cyclic conformational changes cause a rotation of the central stalk. The A₃B₃-headpiece forms a central orifice that is filled by the double α-helical structure of the N- and C-terminus of subunit D. The globular domain of D is interacting with the co-rotating subunit F as well as with subunit C, which is in direct contact to the membrane located A₂O-section [3-5].

Previously, it was shown that the presence of subunit F stimulates the ATPase activity of the A₃B₃DF-complex of the *M. mazei* Gö1 A-ATP synthase [6]. Especially the C-terminal peptide 96GVDLWK₁₀₁ was found to increase the activity two-fold as well as the N-terminal peptide ₁MELA₄, while the truncation mutant F₁₈₇ showed almost no ATPase activity. In addition, the mutation R₈₈L decreases the activity by 20%. Subunit F (101 residues) exhibits
a two domain structure in solution. Its 78 N-terminal residues form a globular domain consisting of a four-stranded β-sheet with a hydrophobic surface that is interacting with the positive charged globular domain of subunit D [7]. The C-terminal domain (CTD) forms a flexible (Fig. 1B; [7]), positively charged tail [7,8] that can move up and down relative to subunit B (Fig. 1B). [7-10]. In the complete A₁A₀-ATP synthase the C-termi of subunits B and F can form a cross-link [8,11] when subunit F is in the extended form.

To study the flexibility of the CTD of subunit F in the active complex we labelled residue F₈₇ near the C terminus of subunit F and residue D₇₁ in the globular domain of subunit D with two different fluorescent dyes. In a confocal microscope setup we observed the fluorescence of single molecules (sm) diffusing through the focus. After laser excitation of the donor dye on D₇₁, instead of emitting the energy as fluorescence light, the energy can be transferred non-radiatively directly to the acceptor dye on F₈₇, which then in turn emits the light as fluorescence, but at a longer wavelength. The efficiency of this Förster resonance energy transfer (FRET) depends mainly on the distance between the donor and acceptor dye as described by T. Förster [12,13]. We measured the fluorophore distances within single A₃B₃DF molecules one after another [14,15]. The approach is called single-molecule FRET (smFRET) and allows to quantify and compare distance distributions counting many single molecules but without FRET averaging. Small sub-populations of A₃B₃DF complexes with distinct FRET efficiencies can be detected only by smFRET. Thereby, we revealed a nucleotide-dependent movement of the CTD of subunit F relative to subunit D.

2. Material and Methods

2.1 Cloning of the ABD-fragment and of subunit F₈₇C

The plasmid coding for the M. mazei Gö1 A₃B₃D-complex was generated from the plasmid containing the gene sequence ahaA, ahaB, ahaF and ahaD (for subunits A, B, F and
D, respectively. This plasmid was kindly provided by Prof. Volker Müller, Johann Wolfgang Goethe University, Frankfurt, Germany, in the expressing vector pGEM-4Z [2] by deleting ahaF. All intrinsic cysteines were replaced in subunits A and B (A_{C36V}, A_{C72V}, A_{C181A}, A_{C380V}, B_{C67S}), except for the conserved A_{C263}. A cysteine was introduced in subunit D (D_{A71C}) for fluorescence labelling and a His8-tag at the N-terminus of subunit A for purification. The deletion was performed by overlapping PCR in three steps. First, a fragment corresponding to the terminal region of subunit B (fragment I) was generated by using the forward primer \(a\) (5' - ATTGCCATGGCTCAGCAGACGTAAAACCAAC-3') carrying the intrinsic XhoI site, and the reverse primer \(b\) (5' - GGTCCTCGTAGGCATCAGCAGTACAATCAATATATGAATTTGTTCCG-3') that was complementary mainly to the terminus of subunit B and to a few nucleotides of the start region of subunit D. Second, the entire subunit D (fragment II) was generated by using the forward primer \(c\) (5' - CGGAACAAATTCATATATTGATTGTACTGCTGATGCCTACGAGGACC-3'), which was overlapping with the start of subunit D and primer \(b\), and the reverse primer \(d\) (5' - ATTCGAGCTCGGTACCTTAATCTCTCATTCTTGCGGACTCTCT-3') that carried the SacI site at the end of subunit D. In both PCRs the entire ABFD-fragment served as a template. Equimolar ratios of fragments I and II, with overlapping ends, served as templates in a third PCR with forward primers \(a\) and reverse primer \(d\), which resulted in the ligated product of subunit B and D (fragment III). Fragment III was double digested with XhoI and SacI for 2 h at 37 °C and ligated into the XhoI and SacI digested ABFD-fragment, where a part of subunit B and the entire subunits F and D were deleted, resulting in a plasmid containing the ABD-fragment.

Subunit F was cloned into the pET-9d1 vector [16], and a cysteine (L87C) was incorporated using the forward primer 5' -
GCTCATGCCATGGAGTTAGCAGTGATCGGAAAGAGCGAATTC-3’ and the reverse primer
5’-GCCGAGCTCTTTACTTCCACAGATCAACACCTACCGCTTTATTATTTTCTCTACAACCTCGTTGAGCC-3’ in a standard PCR reaction.

2.2 Protein purification

The *M. mazei* Gö1 A3B3D-complex was expressed in *E. coli* DK8 according to Singh et al. [6] and subunit F\textsubscript{L87C} was produced in *E. coli* BL21 (DE3) cells as described in [7]. Purification of the labelled products was performed according to [6].

2.3 Protein labelling

For labelling of residue D\textsubscript{A71C} of the *M. mazei* Gö1 A3B3D-complex with ATTO 488-maleimide, 1.8 μM of A3B3D-complex was incubated with 1.35 μM of ATTO 488-maleimide dye in buffer A (50 mM Tris/HCl pH 7.5, 200 mM NaCl, 10% glycerol) for 1 min on ice. The protein was separated from the free dye by 3x washing in a centrifuge filter (MW Exclusion size: 100 kDa). The resulting degree of labelling, i.e. the molar ratio of fluorophore to protein, was 0.72, with A\textsubscript{C263} labelled partly. 250 μM of subunit F\textsubscript{L87C} in buffer A (50 mM Tris, pH7.5, 100 mM NaCl) was incubated for 30 min with 1 mM TCEP (Tris(2-carboxyethyl)phosphine) prior to labelling with 1.3x ATTO 647N-maleimide for 10 min on ice. Labelled subunit F was purified on a Superdex 75 column with buffer A. The degree of labelling was about 1. Labelling reactions were stopped by adding 1 mM of N-acetylcysteine.

2.4 Custom-designed confocal microscope for smFRET

The variable design of the confocal microscope setup has been described previously [17-19]. Here, a ps-pulsed laser (PicoTA 490, Picoquant, Germany) as used to excite the FRET
donor fluorophore Atto488 with 150 µW at 488 nm using a repetition rate of 40 MHz. A second pulsed laser (LDH 635B, Picoquant, Germany) was used for pulsed duty-cycle optimized alternating excitation [20] of the FRET acceptor dye Atto647N with 30 µW at 635 nm, and was delayed by 14 ns with respect to the preceding 488 nm pulse. Diameters of both co-aligned lasers were adjusted to 1 - 2 mm. A dual-band dichroic beam splitter (HC Dual Line 488/633-638, AHF, Tübingen, Germany) directed the lasers through a 60x water immersion objective (UPlanSApo 60xW with N.A. 1.2, Olympus, Germany), and separated scattered laser light from fluorescence photons. After an 150 µm pinhole, fluorescence was separated into two channels by a dichroic beam splitter (zt RDC 640, AHF). FRET donor fluorescence was detected between 500 and 570 nm after an additional band pass filter (ET 535/70M, AHF) and FRET acceptor fluorescence was detected for wavelengths $\lambda > 647$ nm after a combination of two long pass filters (Edge Basic LP 635 and Razor Edge LP 647 RU, AHF) using two single photon-counting avalanche photodiodes (SPCM-AQR-14, Perkin-Elmer). Arrival times of photons were recorded with synchronized TCSPC electronics (SPC-154, Becker & Hickl, Berlin, Germany). FRET data analysis was performed with the software 'Burst Analyzer' (Becker & Hickl, Berlin, Germany).

2.5 ATP hydrolysis assay

A continuous ATP hydrolysis assay was applied to measure the specific ATPase activity of the reconstituted A$_3$B$_3$DF-complex. In this assay, ATP was constantly regenerated by an enzymatic reaction, while the consumption of NADH was measured spectroscopically at 340 nm. The change in absorbance was measured for 250 s in 1 s intervals at 37 °C after adding 5.2 µg of protein complex to 1 ml reaction solution (100 mM Tris/HCl pH 7.5, 25 mM KCl, 4 mM MgCl$_2$, 2.5 mM phosphoenolpyruvate, 2 mM ATP, 0.4 mM NADH, 16 units L-lactic
acid dehydrogenase, 18 units pyruvate kinase), and its activity was derived by fitting the initial linear section of the slope.

3. Results

3.1 Protein purification and labelling

Previously it was established that the CTD of subunit F is stimulating the ATPase activity [6]. In order to communicate structural alterations of subunit F with the catalytic A-B interfaces inside the A₃B₃DF-complex, subunit F has to come in close proximity to the catalytic centre with its C-terminus. To prove whether such movements occur, smFRET-experiments were designed. A mutant *M. mazei* Gö1 A₃B₃D was generated, where all internal cysteines in subunits A and B were mutated (except the conserved C₂₆₃ in subunit A). In addition, a cysteine was introduced at the tip of subunit D (D₄₇₁C) for fluorescence labelling (Fig. 2A). For simplicity, this complex will be subsequently referred to as the *MmA₃B₃D*-complex. A mutant of subunit F contained a cysteine at position 87 (F₈₇₇C). Based on our model (Fig. 2A) the distance of the cysteines was estimated to be in the range of 4 nm for efficient energy transfer. The purified *MmA₃B₃D*-complex was labelled with the donor dye ATTO 488-maleimide with 72% efficiency and the purified subunit F was labelled with the acceptor dye ATTO 647N-maleimide with almost 100% efficiency. Specific labelling was confirmed by a fluorescence image of the respective SDS-gel (Fig. 2B).

3.2 Measuring smFRET with *MmA₃B₃DF* in solution

The FRET-labelled *MmA₃B₃DF*-complex was formed after stoichiometric addition of ATTO 647N-labelled subunit F to ATTO 488-labelled *MmA₃B₃D* and incubation for 30 min at room temperature. After reconstitution the ATPase activity of the labelled *MmA₃B₃DF*-complex was measured and compared to the labelled *MmA₃B₃D*-complex. For the
reconstituted labelled complex we found a three-fold higher specific ATPase activity (22 ± 0.5 nmol min⁻¹ (mg protein)⁻¹) than for the labelled complex without labelled subunit F (7 ± 0.7 nmol min⁻¹ (mg protein)⁻¹). This ratio is in line with our previous results, where we showed that subunit F stimulated the ATPase activity of the complex three-fold [6], and indicates that the reconstituted complex was formed. Immediately before the smFRET recordings, the MmA₃B₃DF solution was diluted to 1 nM in buffer B (50 mM Tris, pH 7.5, 100 mM NaCl, 4 mM MgCl₂). A 50 µl droplet of this protein solution was placed on a glass coverslip, and the alternating lasers were focused 100 µm deep into the solution. Fluorescence photons were registered when labelled proteins diffused through the detection volume (Fig. 3A). After each 1000 s measurement section, the solution had to be replaced due to quick binding of MmA₃B₃DF-complexes to the glass surface. FRET-labelled MmA₃B₃DF-complexes were identified as photon bursts in the photon time traces by an automated search applying intensity thresholds for both FRET excitation with 488 nm as well as FRET acceptor fluorophore control with 635 nm. In addition, the minimal burst length was set to 5 ms, i.e. ten times longer than the average diffusion time of a single fluorescent impurity.

An excerpt of a FRET time trace is shown in Fig. 3B. In the presence of 1 mM Mg-ATP in buffer B photon bursts with different relative intensities of FRET donor on MmA₃B₃D (blue trace, I₀) and FRET acceptor (green trace, Iₐ) on subunit F within a single burst were found. For example, the first MmA₃B₃DF-complex appearing around measurement time t = 70 ms showed similar intensities I₀ and Iₐ for excitation with 488 nm. A comparable intensity was recorded for the directly excited FRET acceptor (red trace) with 635 nm. The next A₃B₃DF-complex at t = 120 ms exhibited significantly distinct intensities for I₀ and Iₐ. For all photon bursts we calculated the simplified equivalent of a mean FRET efficiency, i.e. the proximity factor P, using the equation:
\[ P = \frac{I_A}{(I_D + I_A)} \]

As shown in Fig. 3B, the first \( \text{MmA}_3\text{B}_3\text{DF} \)-complex exhibited a proximity factor \( P = 0.47 \), whereas the second complex had \( P = 0.28 \). A third \( \text{MmA}_3\text{B}_3\text{DF} \)-complex in the time trace at \( t = 120 \text{ ms} \) showed similar FRET donor signal intensity, but a lower proximity factor \( P = 0.09 \).

We also found \( \text{MmA}_3\text{B}_3\text{D} \)-complexes without an attached labelled subunit F (bursts at \( t = 200 \text{ ms} \)). These complexes were characterized by a fluorescence from the FRET donor fluorophore ATTO 488 only (blue traces). Here, we could not discriminate whether an unlabelled but not detectable F was bound or not. In contrast, the next photon burst in the time trace at \( t = 360 \text{ ms} \) was a \( \text{MmA}_3\text{B}_3\text{DF} \)-complex without a FRET donor fluorophore on D, and is characterized by a fluorescence of the directly excited FRET acceptor solely (red trace, excitation with 635 nm pulses). At the end of this except we found another FRET-labelled \( \text{MmA}_3\text{B}_3\text{DF} \)-complex with \( P = 0.54 \) at time \( t = 550 \text{ ms} \).

3.3 FRET histograms in the presence of different Mg-Nucleotides

The FRET efficiency or the proximity factor \( P \), respectively, depends on the distances between the fluorescent dyes and decreases with increasing inter-fluorophore distance. We analysed the FRET efficiencies for four different biochemical conditions: in the presence of Mg-ATP, Mg-ADP, Mg-AMPPNP and in the absence of added nucleotides in buffer B as a control. The resulting proximity factor histograms are summarised in Figure 4.

In the absence of nucleotides, i.e. in buffer B containing 4 mM MgCl\(_2\), the proximity factor distribution is broadened with a maximum between 0.4 and 0.7 (Fig. 4A). Given comparable fluorescence quantum yields of 0.8 for ATTO 488 and 0.65 for ATTO 647N, a Förster radius of 4.9 nm for this FRET pair (according to the supplier ATTO-TEC) and similar detection efficiencies for FRET donor and acceptor fluorescence in our setup, a
proximity factor around 0.5 corresponds to a distance of about 5 nm, which is in good agreement with the structural model shown in Figure 2A.

In the presence of Mg-ATP (Fig. 4B), the proximity factor distribution changed and was broadened. A second peak with a lower P between 0.1 and 0.25 was determined. However, also the relative amount of the MmA3B3DF-complex with P > 0.7 increased. P values lower than 0.3 corresponded to a distance increase between the FRET-labels ATTO 488 at the tip of subunit D and ATTO 647N at the C-terminal region of subunit F, whereas P values larger than 0.7 indicated a shorter distance between the C-terminus of subunit F and the tip of subunit D. In comparison, addition of Mg-ADP (Fig. 4C) did not change the proximity factor distribution as found for buffer B only. Also in the presence of Mg-AMPPNP (Fig. 4D) the proximity factor distribution appeared to be very similar to the case of buffer B only, but a minor additional population of MmA3B3DF-complexes with low P was recognized.

4. Discussion

In A-ATP synthases and the related bacterial V-ATPases like Thermus thermophilus subunits A3B3D form the minimal complex capable of hydrolysing ATP [21,22]. However, subunit F from M. mazei Gö1 (F_{Mm}) increases the ATPase activity of the MmA3B3DF-complex 4x [6], and the related subunit F from T. thermophilus (F_{Tt}) increases the torque of the rotating A3B3DF-complex 1.6x [23]. Especially its CTD is important for the increase, as A3B3DF-mutants, where the CTD of subunit F was deleted, showed an even lower ATPase activity or torque as the respective A3B3D-complex alone. It has been reported that the CTD of subunit F is flexible and can exist in a retracted or extended form [7]. F_{Tt} can switch from the retracted to the extended form in the presence of Mg-ATP [24]. Our results from the nucleotide-dependent FRET measurements, revealing that 2 mM Mg-ATP can induce a low FRET state in the MmA3B3DF-complex, corroborate this finding. This indicates that the CTD
of subunit \(F_{Mm}\) has moved away from the globular domain of subunit D, which is equivalent to forming the extended conformation. In contrast, Mg-AMPPNP, Mg-ADP, or Mg\(^{2+}\)-containing buffer alone cannot induce this conformation, and subunit \(F_{Mm}\) remains in the retracted conformation, which resulted in a high FRET signal only. The fact that both FRET states existed in the presence of Mg-ATP is in line with the idea that subunit \(F_{Mm}\) moves up and down in the \(MmA_3B_3DF\)-complex during ATP hydrolysis.

A model based on crystal structures of the bacterial ATPase from \textit{Enterococcus hirae} proposes that subunits DF rotate in 120° steps and the ATP-binding and catalysis events are combined in a single dwell between 120°-steps, while ADP-release forces the rotation of the central stalk by 120° [25]. The crystals that represent the catalytic dwell contained two Mg-AMPPNP or no nucleotides, crystals that represent the ATP-binding dwell contained two Mg-ADP, and the ADP-release dwell was inferred from crystals with three Mg-ADP. In all three structures subunits DF have the same rotational orientation (only a tilting movement was observed), and subunit F is not extended. This is in line with our results in solution where we did not observe a low FRET state in the presence of Mg-AMPPNP, Mg-ADP or Mg\(^{2+}\)-containing buffer only. Therefore, the small fraction of the low FRET state that occurred in the presence of MgATP is a transient state during ATP hydrolysis that is so far not represented by a crystal structure.

A-ATP synthases are composed of two rotary engines, including the membrane-embedded \(A_O\)-complex (\(ac\)), and the \(A_3B_3CDF\)-complex [4,5]. Both motors are connected by two peripheral stalks each composed of an EG-heterodimer. These complexes share common features with bacterial \(F_1F_O\) -ATP synthases; \((F_1: \alpha_3\beta_3\gamma\delta\epsilon\text{ and } F_O: ab_2c_9\)) [26,27]. In both enzymes the motors are connected by a central stalk that serves as an axle, as well as by one (F-ATP synthases) or two (A-ATP synthases) peripheral stalks. The minimal subunit composition of the F-type rotor during ATPase-driven rotation is the \(\gamma\)-subunit, composed of
a coiled-coil domain and a globular domain known as the foot [2,23,28]. Based on structural studies it has been proposed, that the A-ATP synthase subunits D and F assemble in a manner similar to the coiled-coil and foot domains of subunit γ [31], reflecting also the structural difference of the conserved β-barrel structure of the NTD of bacterial subunit ε [30,31] compared to the alternating β-sheet and α-helix structure of the NTD in the A-ATP synthase subunit F [7]. However, like subunit F of A-ATP synthases, the rotary subunit ε of bacterial F-ATP synthases is composed of a globular NTD and a CTD [32]. The latter consists of a helix-loop-helix motive, which can likewise exist in a compact (εc) [32-34] or extended (εe) conformation [32-35]. The very C-terminal helix 2 in the εe state interacts with the subunits α, β and γ [32-35]. For some bacterial enzymes it was shown that the CTD of subunit ε has an ATP binding motive that can act as an ATP sensor [36,37]. Similarly, Raghunathan et al. proposed a model for the M. mazei Gö1 A-ATP synthase, where ATP can bind to the CDT of subunit F. In this model subunit F_{Mm} facilitates the binding of ATP to the nucleotide-binding site in subunit B [10]. In contrast, unlike the stimulating role of subunit F the CTD of subunit ε in its extended conformation was shown to be an intrinsic inhibitor for ATP hydrolysis, but not for ATP synthesis, to prevent futile ATP hydrolysis at low ATP concentrations [37]. Despite similar domain components subunits F and ε have very different regulatory functions in their respective ATP synthases. It remains an open question how these functions evolved and how they can help their host to adapt to the local environment.
References


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Figure legends

Fig. 1. (A) Subunit and domain arrangement of the $A_1A_0$ ATP synthases. The NMR structure of the *M. mazei* Gö1 subunit F ([7]; PDB ID 2OV6) is shown in *magenta*. The structurally unknown region of subunit a (345-668) is shown as yellow cylinder. As shown for the $A_3B_3DF$-complex of the $A_1A_0$ ATP synthase from *M. mazei* Gö1 [38] and *M. jannaschii* [8], respectively, the C-terminus of subunit F forms a cross-link with the C-terminus subunit B (*green*) in the presence of MgATP. The C-terminal peptide $88\text{RERKIK}_92$ of subunit F and $390\text{EALSERDTK}_399$ of subunit B were identified by MALDI TOF [8], and are indicated by sticks. For clarity only the A:B:F-front side of the $A_3B_3$-headpiece is shown. (B) Based on NMR correlation time and relaxation measurements it has been shown that the CTD of the *M. mazei* Gö1 subunit F experiences conformational mobility [7].

Fig. 2. (A) A model describing the arrangement of subunits A, B, D and F in a complex with respect to the smFRET experiment. The model was generated using PyMOL [39], by assembling the three dimensional structures. Subunit A is highlighted in *orange*, B in *green*, D in *yellow* and F in *magenta*. The substituted residues are labeled and shown as a sphere. The distance between residues D-A71C and subunit F-L87C is shown by a dashed line and calculated to be 4 nm, which is in the permissible range for a FRET experiment. The residues of subunits B and F involved in B-F crosslink formation [8] are indicated. (B) The SDS-gel shows *M. mazei* Gö1 $A_3B_3D\text{-ATTO 488}$ in *lane 2*, *lane 1* is the protein ladder and *lane 3* represents a fluorescence scan (488 nm) of *lane 2*. *Lane 3* shows a dark band for labelled D$\text{A71C}$ and few light bands above D$\text{A71C}$ for some impurities. (C) 17% SDS gel of purified $MmF\text{-L87C-ATTO 647N-maleimide (lane 1)}$, and fluorescence scan of *lane 1* (*lane 2*; wavelength of 647 nm). The dark band represent the monomeric subunit F mutants. *Dimer*, indicate a small amount of dimeric $F_{Mm}\text{-L87C}$ and LM represents a protein ladder.
Fig. 3. (A) Confocal smFRET setup with diffusing $MmA_3B_3D$-complex, labelled with ATTO 488 in subunit D (blue dot) and ATTO 647N on subunit F (red dot). (B) Confocal fluorescence time trace with individual photon bursts of FRET-labelled $MmA_3B_3D$ complexes in the presence of Mg-ATP recorded for 600 ms. In the lower panel FRET donor intensity (ATTO 488) is shown as blue trace and the FRET acceptor intensity (ATTO 647N) is shown as green trace following pulsed 488 nm excitation. The FRET acceptor intensity by interleaved pulsed excitation with 635 nm is shown as red trace. Three photon bursts highlighted by yellow boxes exhibit different proximity factors as indicated in the upper panel. Photon bursts of complexes with only one fluorophore (donor only or acceptor only) are indicated also.

Fig. 4. Histograms of the proximity factor for FRET-labelled $MmA_3B_3D$ complexes in the presence or absence of different nucleotides. (A), the proximity factor for FRET-labelled $MmA_3B_3D$-complexes was determined in buffer only, (B) in the presence of 1 mM Mg-ATP, (C) in the presence of 1 mM Mg-ADP, or (D) in the presence of 1 mM Mg-AMPPNP. The gray areas indicate the low ($P = 0.1 - 0.25$) and high ($P = 0.4 - 0.7$) FRET zones, respectively.
Figures 1A-B
Figures 2A-C
Figures 3A-B