Three-stepped rotation of subunits γ and ε in single molecules of F-ATPase as revealed by polarized, confocal fluorometry

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Abstract The proton translocating ATP synthase is conceived as a rotatory molecular engine. ATP hydrolysis by its headpiece, CF_1, drives the rotation of subunit γ relative to the hexagonally arranged large subunits, $(\alpha\beta)_3$. We investigated transition states of the rotatory drive by polarized confocal fluorometry (POCOF) as applied to single molecules of engineered, immobilized and load-free spinach-CF_1. We found that the hydrolysis of ATP caused the stepped and sequential progression of subunit γ through three discrete angular positions, with the transition states of γ being too shortlived for detection. We also observed the stepped motion of ε, whereas δ was immobile as $(\alpha\beta)_3$.

Key words: ATP synthase; Single molecule; Rotation; Molecular motor

1. Introduction

The proton translocating ATP synthase is conceived as a rotatory molecular engine [1–3]. In the isolated headpiece, F_1, and under conditions of ATP hydrolysis the rotation of subunit γ relative to the hexagonally arranged large subunits, $(\alpha\beta)_3$, has been established experimentally by chemical cross-linking [4], by polarized absorption recovery after photobleaching [5] and by video-microfluorometry [6]. In contrast to the first technique, the latter two are time-resolving (see [3] for a review) with the following virtues and drawbacks: The about 2 μm long fluorescent actin filament in [6] allows the direct observation in the microscope of the *unidirectional* rotation of subunit γ in single molecules of F_1. Because the filament represents a heavy viscous load on subunit γ the maximum torque of this motor has been revealed, but finer motional details were damped out. The photoselection technique in [5], on the other hand, operates with a single dye molecule on subunit γ, i.e. at a negligible load, but the ensemble averaging hinders a rigorous discrimination between random and unidirectional rotation in some special cases. We treated this difficulty by developing a theory of molecular stepping motors [7] that has led to the proposal that the data in [5,8] might indicate a three-stepped rotation of subunit γ. In the present article we describe a new technique, polarized confocal fluorometry (POCOF), that overcomes the remaining ambiguity by combining the benefit of single-molecule spectroscopy with the one of a low load on the enzyme. Within noise limits it revealed a three-stepped rotation of subunit γ and a first assessment of the residence times of subunit γ in the three metastable and the more transient interstitial angular positions (transition states).

2. Materials and methods

A photon-counting fluorescence microscope, the ConfoCor (Carl Zeiss Jena/Evotec, Hamburg, Germany), was used to investigate transients of the angular position of the fluorescent probe on a single molecule of immobilized F_1. The instrument counts fluorescence quanta emitted by single molecules contained in a very small volume element ($< 10^{-15}$ l) as defined by the focus of a laser beam (diameter ~0.5 μm). Fig. 1B schematically illustrates the focus of the ConfoCor with a γ-labelled molecule of F_1 immobilized at the glass/water interface of a chamber slide.

The headpiece of the ATP synthase from spinach, CF_1 (for chloroplast F_1), was covalently and specifically labelled by a fluorescent probe, a maleimide derivative of tetramethylrhodamine, TMR. The target cysteine residue was located either on subunit γ, ε or δ. The particular residue on γ (C322) was intrinsic [9,10] whereas the residues on ε (S85C) and δ (S10C) were engineered [11,12]. In the latter two cases the engineered subunits were first labelled and then reassociated with unlabelled $CF_1(-\delta, \varepsilon)$. The labelling did not impair the ATPase activity of solubilized CF_1. Labelled and solubilized CF_1 was immobilized by adsorption at the water/glass interface of a chamber slide. Fig. 1A shows the respective positions of the label in a current structural model of CF_1. Fig. 2 shows the selective modifications of the subunits as detected by the fluorescence of the bound dye.

2.1. Preparation of CF_1 and labelling

CF_1 lacking subunits δ and ε ($CF_1(-\delta, \varepsilon)$) was prepared by anion exchange rechromatography [13] of CF_1 prepared from EDTA extracts of spinach chloroplasts [14]. The specific activity of the enzyme was 23 U/mg (measured in 50 mM Tris/HCl, pH 7.8, 5 mM ATP, 2 mM $MgCl_2$, 20% (v/v) methanol, 10 mM Na_2SO_3, 5 min, room temperature). $CF_1(-\delta, \varepsilon)$ was specifically labelled at γ-Cys-322 by treatment at room temperature for 10 min at pH 7.0 (25 mM MOPS/NaOH) with 20 μM tetramethylrhodamine-5-maleimide (TMR-5-M, Molecular Probes, Eugene, OR, USA). The reaction was terminated by addition of 1 mM *N*-acetyl-cysteine. Free dye was separated from labelled protein by gelfiltration (Pharmacia NAP5/25 mM Tris/HCl, pH 7.8). Recombinant subunits δ (S10C) and ε (C6S, S85C) were over-expressed in *E. coli* [15] and purified by anion exchange and hydrophobic interaction chromatography (δ) [11] or cation exchange chromatography (ε) [12]. Both proteins were labelled with tetramethylrhodamine-5-maleimide similarly as $CF_1(-\delta, \varepsilon)$ but at 200 μM TMR and overnight. $CF_1(-\delta, \varepsilon)$ was substituted with either δ or ε or both subunits by addition of molar amounts of δ and a 6-fold molar excess of ε. In the latter case care was taken to keep the total amount of urea below 800 mM (ε was dissolved in 8 M urea). All samples ($CF_1(-\delta, \varepsilon)$+TMR-γ, $CF_1(-\delta, \varepsilon)$+TMR-δ, $CF_1(-\delta, \varepsilon)$+TMR-ε) were gelfiltrated through Pharmacia Superose-12 immediately prior to measurements in the ConfoCor. The column was cleaned by 6 M guanidinium-HCl weekly. Samples (100 μl), irrespective of the content of small subunits, eluted at $V_e = 9.6$ ml. The specific Mg-ATPase activities remained unchanged upon labelling of either subunit γ or after substitution with TMR-δ or TMR-ε. Ca-ATPase activity was decreased from ~20 U/mg to 4 U/mg upon substitution with TMR-ε as expected.

2.2. The immobilization of labelled CF_1

The immobilization of labelled CF_1 by adsorption to the glass-

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buffer interface was monitored by fluorescence autocorrelation spectroscopy, FCS, with the exciting light beam of the ConfoCor focussed into the solution, about 200 μm above the surface of the chamber slide. The concentration was determined by fluorescence autocorrelation analysis using the algorithms, hard- and software by Evotec (Hamburg, Germany). For immobilization of CF_1 a droplet containing 30 μl of 5 nM CF_1 lacking subunits δ and ε and labelled with TMR-5-M within subunit γ ($CF_1(-\delta, \varepsilon)$+TMR-γ) in the presence of a 100-fold excess of unlabelled enzyme was deposited on the chamber slide. It contained 25 mM Tris/HCl, pH 7.8 and either 1 mM Mg-ADP in case of the inhibited enzyme or 20% (v/v) methanol for successive measurements under hydrolyzing conditions. After about 1 min the solution was removed from the slide, exchanged by the respective enzyme-free buffers and left to settle for another 10 min. After this procedure, there were no free CF_1 molecules detectable in the focus spot at about 200 μm depth in the buffer, indicating that the immobilization of the enzyme by adsorption to the glass/water interface was complete.

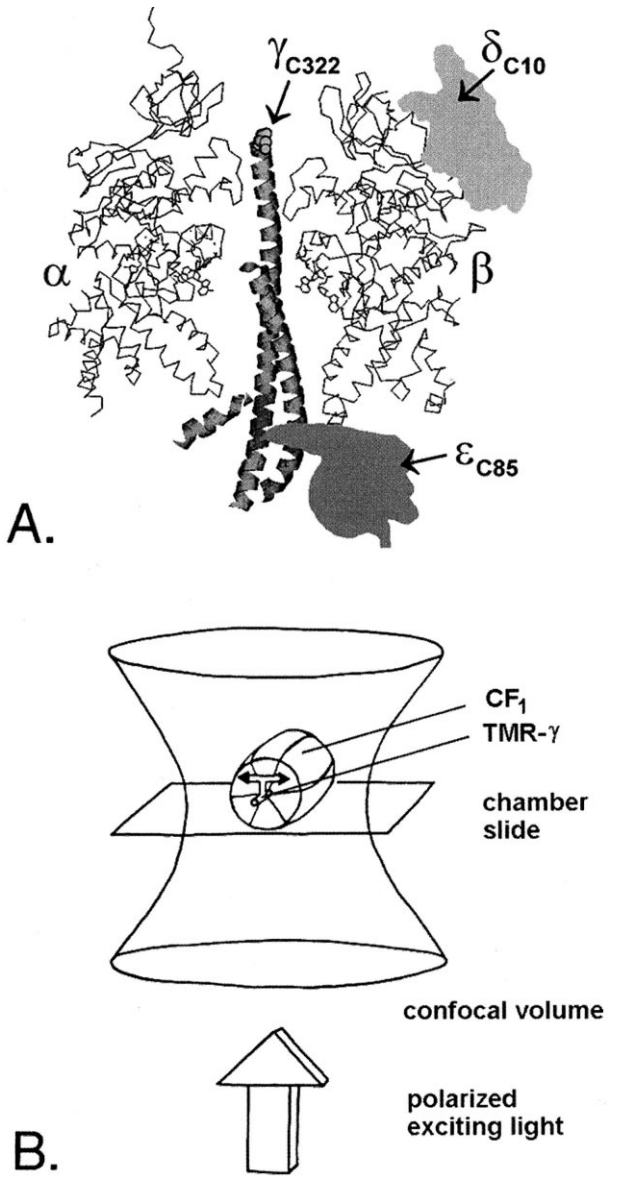

Fig. 1. A: A current *model* of CF_1 [21]. The arrows indicate the respective cysteine residues in subunits γ, δ, and ε that were modified with TMR-5-M. B: The focal volume in polarized confocal fluorometry. The barrel on the surface of the chamber slide denotes immobilized and γ-labelled F_1 (not drawn to scale). The arrow denotes the exciting laser beam and its plane the E-vector of the light.

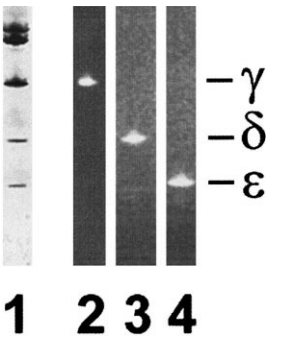

Fig. 2. SDS-gel of CF_1 demonstrating by fluorescence the specific labelling of subunits γ and δ and ε, respectively.

2.3. Intersubunit rotation

Intersubunit rotation was detected with the exciting light beam directly focussed onto the surface of the chamber slide. TMR was excited by continuous linearly polarized focussed argon ion laser, 514 nm (10 mW). Because of the linear polarization of the exciting light, the count rate varied as a function of the orientation of the dye relative to the polarization vector of the beam. This holds true even though the bound dye molecule carries out rapid rotational diffusion (in nano- to microseconds) around its bond axis relative to the protein. This motion diminishes the polarization anisotropy only gradually as demonstrated elsewhere [8].

3. Results and discussion

In a first set of experiments the laser focus was positioned into a droplet containing solubilized CF_1 which was deposited on a chamber slide. The concentration of labelled CF_1 was determined by the ConfoCor operating in the FCS mode (fluorescence correlation spectroscopy) [16]. To avoid too tight a packing of labelled molecules at the interface, we used a 100-fold dilution of labelled in unlabelled CF_1 molecules in the starting solution. The specific Mg-ATPase activity of *immobilized* CF_1 was decreased 10-fold in comparison with the one of the solubilized enzyme.

After 10 min of incubation the laser focus was readjusted to hit immobilized CF_1 at the glass/water interface. The ConfoCor was then used in a new mode, coined POCOF. The principle of POCOF, which stands for polarized confocal fluorometry, is illustrated in Fig. 1B. When the laser beam impinges on the immobilized enzyme molecule in the double-conical focal volume, the angular position of the transition moment of the probe (shown as a double arrow) relative to the linear polarization (flat arrow) of the laser beam determines the probability of excitation ($\cos^2(\alpha)$ law) and thereby the rate of emission of fluorescence quanta. This is why the rotation of the label is expected to cause transients of the fluorescence count rate. Such transients were recorded over an illumination interval of about 5 s. Within this period of time the dye molecule in the focus was irreversibly bleached. The laser beam was then shut off, the focus was horizontally shifted (typically by about 10 μm), and the shutter was reopened for a recording at another spot containing another molecule of labelled CF_1 with a 'fresh' dye molecule. The light stability of rhodamines, the favored dyes in such studies, is of the order of 10^5–10^6 absorbed quanta [17].

3.1. Transients with γ-labelled CF_1

Transients with γ-labelled CF_1 are depicted in Fig. 3. Fig. 3A shows the typical result when the hydrolysis activity was

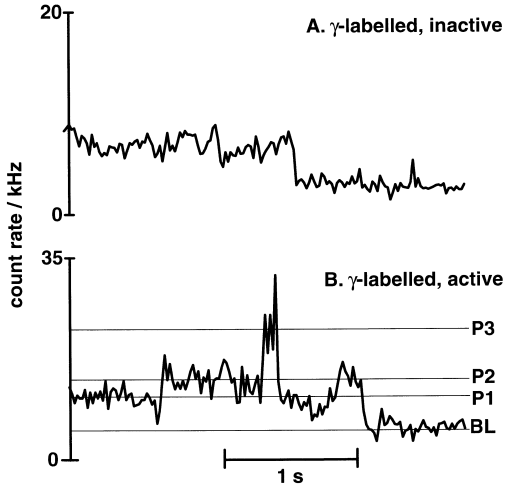

Fig. 3. Transients of fluorescence upon excitation with linearly, polarized light of a single, γ-labelled and immobilized molecule of $CF_1(-\delta, \varepsilon)$. A shows a typical transient under inhibition of the enzyme by ADP. B shows active CF_1 in the presence of ATP: after adsorption of the enzyme to the chamber slide and a 10 min incubation period in the presence of 50 μl 25 mM Tris/HCl, pH 7.8 and 20% (v/v) methanol, 450 μl reaction mixture for ATP hydrolysis (50 mM Tris/HCl, pH 7.8, 5 mM ATP, 2 mM $MgCl_2$, 20% (v/v) methanol, 10 mM Na_2SO_3) were added to the sample. Note the three distinct levels (P1 to P3) of the count rate of fluorescence (as implied by the three thin lines) which indicate three discrete angular positions of the labelled γ relative to $(\alpha\beta)_3$ during the catalytic cycle. The final level, BL, results from the bleaching of the molecule in the very focus.

blocked by the presence of ADP. At zero time, when the photoshutter was opened, the count rate started at a level of 5 kHz. It continued at this level over about 1.5 s, to suddenly break-down to a background level of about 2 kHz. From there on the count rate continuously decayed over more than 10 s. We attributed the count rate of 3 kHz over a background of 2 kHz to a particular single molecule of CF_1 in the very focus and the sudden break-down to the irreversible bleaching of the covalently attached TMR molecule (see [17] for a study on the light stability of TMR). The slower and gradual bleaching of the background level was attributed to molecules in a more unfavorable position at the fringes of the focal volume. The constancy of the count rate until the sudden bleaching occurred, demonstrated that subunit γ was not rotating under these conditions. This interpretation was corroborated by two observations: (1) When the same focal spot was reilluminated after a period of darkness, the high count rate was never revived. (2) When the microscope table was laterally moved to bring a 'fresh' molecule into focus, high count rates with a sudden break-down were observed again. This pattern of a high count rate to start with and a sudden drop to a lower level was observed in over 200 shots with γ-labelled CF_1. Under the same conditions we never observed a sudden rise of the count rate. The only other behavior was a background rate right from the beginning because there was no favorably oriented, labelled enzyme in the focus.

Fig. 3B documents the completely different behavior when γ-labelled CF_1 was hydrolyzing ATP. The count rate started at a low level, P1, to suddenly raise to a higher level, P2, and by another jump to P3, then dropping down to P1, and further up to P2, again. Then the level dropped to a basal level, BL. The latter was attributed to the background after the bleaching of the particular dye molecule in the very focus. We interpreted this behavior as indicating the stepwise progression of subunit γ through three discrete angular positions, P1, P2, and P3. A stepped motion of subunit γ in the presence of ATP was observed in more than 200 experiments, a clearly three-stepped one only in three. This is not astounding in terms of the angular resolution and the noise limits. It is noteworthy that e.g. two angular positions that are symmetrically placed to the interface are indistinguishable by their resulting count rates. This will be discussed elsewhere. The noise level was strictly proportional to the square root of the counts per time channel. Attributable to quantum or shot noise it was not to be lessened, because of the limited light stability of the dye.

The time base was digitized at intervals of 10 ms. At this time resolution the transients were instantaneous. The number of three steps conformed with the expectation based on (a) the number of three reactive sites on the hexagon formed by the large subunits of CF_1 [1,18], and (b) the interpretation of our previous experiments with a large ensemble of labelled CF_1 [5] in the light of a theory of molecular stepper motors [7]. It is an important new result that the angular positions of the transitions between two sites were so shortlived. Within stochastic limits, the average persistence on levels P1, P2, and P3 was much longer than the transit time between them. It was compatible with the observed average turnover time of 85 ms for ATP hydrolysis by immobilized CF_1.

3.2. Transients of CF_1 labelled at subunits δ and ε

Transients of CF_1 labelled at subunits δ and ε are shown in Fig. 4A and B, respectively. In both cases the enzyme was hydrolyzing ATP. With the label on subunit δ we consistently (200 cases) observed a behavior with a high count rate at the beginning and a sudden drop to a bleached level that was

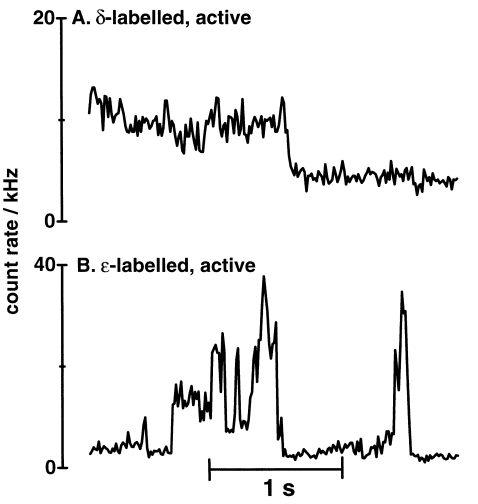

Fig. 4. Transients of fluorescence upon excitation of a single immobilized molecule of CF_1 with linearly, polarized light under ATP hydrolysis (ATP but no ADP added). TMR was covalently attached to either subunit δ (A), or ε (B). All other conditions were as described in Fig. 3. Note that the signal with TMR-labelled ε displays the same major characteristic of the signal recorded with active CF_1 which was labelled in γ. The signal with TMR-labelled δ resembles the signal obtained with γ-labelled but ADP-inhibited CF_1 (cf. Fig. 3A).

attributable to a non-rotating element (see also Fig. 3A). Contrastingly there were uprising count rates when the label was attached to subunit ε. Subunit ε did not reveal the regularity of stepping through three positions as subunit γ. Although the stochastic character of the stepped rotation hampers a direct comparison of the rotations of ε and γ, these data demonstrate that ε belongs to the rotor (with γ) and δ to the stator of the enzyme (with $(\alpha\beta)_3$) (see [21] for an up-to-date structural model). The same assignment has been inferred from the different effects on the ATPase activity of crosslinking of δ with $(\alpha\beta)_3$ [11] and of ε with γ [12,19], respectively.

Which reaction provides the major driving force for a rotational step is a key question to understand the driving mechanism. The hydrolysis of ATP is expected to proceed in the following sequence: binding of ATP, its sequestration ('tight binding'), the hydrolysis of sequestered ATP, and the release of ADP and P_i. Since our experiments were carried out under ATP saturation, the binding of ATP was not rate-limiting and certainly not responsible for the sudden steps. The hydrolysis of ATP by CF_1 is generally conceived as the inversion of ATP synthesis by F_OF_1, and the release of tightly bound ATP as the major energy requiring step [20]. When F_1 operates in the reverse direction the sequestration of (loosely) bound ATP may be the major driving step. It is conceivable that it pulls the DELSEED 'lever' at the 'loose' binding site (see [1]) inwardly towards the crank of subunit γ to click it round to the next angular position.

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