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Domain compliance and elastic power transmission in rotary $F_0F_1$-ATPase

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The 2 nanomotors of rotary ATP synthase, ionmotive $F_0$ and chemically active $F_1$, are mechanically coupled by a central rotor and an eccentric bearing. Both motors rotate, with 3 steps in $F_1$ and 10–15 in $F_0$. Simulation by statistical mechanics has revealed that an elastic power transmission is required for a high rate of coupled turnover. Here, we investigate the distribution in the $F_0F_1$ structure of compliant and stiff domains. The compliance of certain domains was restricted by engineered disulfide bridges between rotor and stator, and the torsional stiffness ($\kappa$) of unrestricted domains was determined by analyzing their thermal rotary fluctuations. A fluorescent magnetic bead was attached to single molecules of $F_1$ and a fluorescent actin filament to $F_0F_1$, respectively. They served to probe first the functional rotation and, after formation of the given disulfide bridge, the stochastic rotational motion. Most parts of the enzyme, in particular the central shaft in $F_1$, and the long eccentric bearing were rather stiff (torsional stiffness $\kappa > 750$ pNnm). One domain of the rotor, namely where the globular portions of subunits $\gamma$ and $\varepsilon$ of $F_1$ contact the c-ring of $F_0$, was more compliant ($\kappa = 68$ pNnm). This elastic buffer smooths the cooperation of the 2 stepping motors. It is located where needed, between the 2 sites where the power strokes in $F_0$ and $F_1$ are generated and consumed.

First evidence for an elastic coupling element in $F_0F_1$ has been provided by simulations of its kinetic properties both under rate limitation by proton transfer in $F_0$ (13) and by catalysis in $F_1$ (14). A first estimate for the torsional stiffness of the elastic element in $EF_0F_1$ has been obtained from recordings of the torque as a function of the angular reaction coordinate (4). A single molecule of $F_0F_1$ has been immobilized with $F_1$ down on a solid support with a fluorescent actin filament attached to the c-ring of $F_0$ to monitor the rotation as driven by the hydrolysis of ATP. Because of the viscous drag on the long filament (length $> 3 \mu$m) the turnover rate is reduced by $> 2$ orders of magnitude compared with the rate without filament. The viscous drag counteracts the enzyme-generated torque, and this bends the filament. As with a spring balance, its curvature was used to determine the torque as a function of the angular position of the c-ring. Although the driving motor, $F_1$, progressed in steps (of $120^\circ$), the torque profile, as sensed by $F_0$, was smooth, and this was attributed to an elastic coupling element with a torsional stiffness of $\sim 60$ pNnm (4). The theoretical treatment of this nanomachine in terms of the Smoluchowski equation of statistical mechanics has revealed the benefit of an elastic buffer, which is required for a high turnover rate under load (see figure 8 in ref. 4 and also ref. 15). Although the existence of an elastic power transmission between $F_0$ and $F_1$ has been established by the above-cited work (4), the magnitude and the distribution of compliant versus stiff elements over the enzyme structure, in particular between central rotor and eccentric stator, has not been determined before. Here, we investigated the torsional stiffness of several enzyme domains by fluctuation analysis, and localized the elastic buffer in the structure of $EF_0F_1$. The domain compliance was investigated by monitoring either via a magnetic bead (diameter typically 1 $\mu$m) or by a short actin filament (length $< 0.5 \mu$m) the thermal rotational fluctuations of an attached fluorescent probe in immobilized single molecules. To attribute the observed overall compliance to specific enzyme domains we stiffened selected portions by disulfide cross-linking through engineered cysteines. The properties of the respective cysteine pairs, their position in the crystal structure and the correlation between the functional halts of the active enzyme, the disulfide-locked conformations, and the published crystal structures have been presented elsewhere (46).


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Results

Magnetically Driven Rotation and Rotational Fluctuations in EF1. Magnetically driven rotation and rotational fluctuations in EF1 were studied as follows. A single molecule of EF1 was immobilized on a Ni-NTA-coated glass plate by an engineered His-tag at the N-terminal end of each of the three copies of subunit β as illustrated in Fig. 1A. A streptavidin-coated hyperparamagnetic bead was attached with a biotin-maleimide function to the rotor via an engineered cysteine in the bulky protruding domain of subunit γ (γK109C). The bead, typical diameter 1 μm, was fluorescence-labeled by biotinylated quantum-dots (Q-dots), allowing for extended observation times without bleaching. A slowly rotating magnetic field (typically 0.1 revolutions per second) was applied (5 mM Mg-ATP present), and the orientation of the bead was videographed in an inverted fluorescence microscope. If the viewing field contained, say 100 beads total, it typically showed 95 rotating ones. Whether or not this was a relevant, enzyme-related rotation was checked as follows. Aside from the above-mentioned cysteine serving to attach the bead, the mutant EF1 contained 2 additional cysteines; they were engineered to form a zero-length cross-link between rotor and stator under oxidizing conditions, placed opposite each other in the homology structure of EF1 to the crystal structure of MF1 (6). The location of 2 cysteine pairs in the enzyme structure is indicated in Fig. 1C. The magnetically driven rotation of the beads stopped after perfusion of the reaction chamber with an oxidant while the rotating field was still on. Fig. 1B shows the percentage of rotating over total beads in the viewing field as function of 3 cycles where the solution over the glass plate was changed from reducing to oxidizing and vice versa. The points in C are experimental, and the lines are the respective fits by a single Gaussian. For details, see Results.

Rotary Fluctuations and the Torsional Stiffness. When a homogeneous, isotropic elastic rod is fixed at 1 end, whereas it is free at the other end, the thermal impact on the rod (the Langevin force) generates a fluctuating torque, $M$. It torsionally strains the free surface by an angle $\phi$ (in radians), such that $M = \kappa \phi$. The torsional stiffness of the rod, $\kappa$, is expressed in units of pNnm. The elastic energy that is transiently stored in the rod is $U = 1/2 \kappa \phi^2$, and the probability, $p(\phi)$, of finding a given deformation angle is given by Boltzmann’s equation

$$p(\phi) = \text{const} \cdot \exp \left( - \frac{U}{k_B T} \right) = (2\pi \sigma^2)^{1/2} \exp \left( - \frac{\phi^2}{2\sigma^2} \right),$$

yielding a Gaussian whose variance, $\sigma^2$, reads:

$$\sigma^2 = k_B T \kappa^{-1}.$$  

If the elastic element is composed of 2 rods of different stiffnesses, $\kappa_1$ and $\kappa_2$, the resulting variance is the sum of the 2 reciprocal stiffnesses, again calibrated in terms of Boltzmann’s constant $k_B T$. 

Fig. 1. Immobilized EF1 with attached magnetic bead (A), the yield of magnetically forced rotation as function of oxidation-reduction cycles (B), and histograms of thermally driven rotational fluctuations (magnetic field off) after the formation of a disulfide cross-link between the rotor and the stator (C). The green and magenta arrows in A indicate the position of the particular disulfide cross-links in EF1. They are encircled in green and magenta, respectively, in C Inset. The arrows in B indicate the time when the solution was changed from reducing to oxidizing and vice versa. The points in C are experimental, and the lines are the respective fits by a single Gaussian. For details, see Results.
The solution from reducing to oxidizing stopped the rotation. The rotational fluctuations persisted and were attributable to fluctuations within the enzyme. The actin filament itself contributed negligibly because of its short length (see ref. 17).

The Inset in Fig. 2B shows the location of the disulfide bridge in 3 mutants, namely (i) aI223Cel.72C (blue), (ii) βD380C γA87C (green), and (iii) aE284CyA276C (red). The probability distributions of the respective rotational fluctuations are shown in matching colors in Fig. 2B. The respective FWHM translated into the following figures for the torsional stiffness in pNnm: (i) 450 (blue), (ii) 59 (green), and (iii) 47 (red). It implied a stiffness of <68 pNnm for the rotor segment lying between the blue and the green disulfide bridges. The stiffness of the segment between the green and the red disulfide bridges was not to be determined precisely here because of the great compliance of the segment farther up toward FO.

The largest stiffness resulted if the e-ring was locked to subunit a (blue in Fig. 2B). The stiffening effect of this particular cross-link showed that the respective detergent-solubilized and surface-attached EFoF1 construct was intact and, in particular, that the attachment of the stator to the rotor was present. This qualifies the previous notion of a dangling stator in this EFoF1 construct (18). The smaller stiffness (450 pNnm) compared with the one observed with magnetic beads for F1 (1,500 pNnm) was attributable to the complement of the e-ring/F-actin construct. It was not caused by the attachment of F1 to the solid support (see data in Fig. 1C).

Torsional Compliance of the Unrestricted, Active Enzyme. The torsional compliance of the unrestricted, active enzyme, e.g., during 1 ATP-waiting dwell before jumping by 120° into the next dwell, was inferred from long trajectories of rotation. Fig. 3A shows a short segment of a stepped rotary trajectory of 2-s duration, and Fig. 3B shows the respective angular probability distribution. The stiffness of the ADP-saturated and thereby intrinsically locked state (orange in Fig. 3B) was 66 pNnm, very much the same as when the DELSEED lever was cross-linked with the rotor (68 pNnm, green data in Fig. 2B). During the ATP-waiting dwells (typically of 100-ms duration, blue in Fig. 3B) the stiffness was κ = 30 pNnm. It implied that the lever motion (occurring during the ATP wait) contributed a stiffness of 50 pNnm.

Torsional Compliance of the Eccentric Bearing. The torsional compliance of the eccentric bearing was investigated in a construct illustrated in Fig. 4A. A cysteine was added to the C terminus of subunit b (bold letters in NCMNKL...). Two cysteines, one on each copy of the more or less parallel helices of subunit b (19–21), served as attachment sites for a Q-dot-doped magnetic bead via the maleimide–biotin link. Fig. 4B shows the probability distribution of the bead without magnetic field (Upper) and with the magnetic field turning steadily at 0.125 revolutions per second either clockwise (blue) or counterclockwise (red) when viewed from the FO side (Lower). The sign of the displacement was chosen positive when moving in the counterclockwise direction. As expected, the rota-

\[ \sigma^2_{comp} = k_B T (\kappa_1^{-1} + \kappa_2^{-1}) = k_B T \kappa_{result}^{-1}. \]  

If, instead of the standard deviation, σ, in radians, the FWHM of the distribution in degrees is read out, the stiffness, κ, reads as follows:

\[ \kappa = 8 \ln 2 \left( \frac{180}{\pi} \right)^2 k_B T (\text{FWHM})^{-2} \equiv 73,700 \text{pNnmFWHM}(\text{FWHM})^{-2}. \]

In summary: (i) The width of rotational fluctuations yields the torsional stiffness calibrated in terms of \( k_B T \). (ii) In composite rods, the reciprocal stiffnesses are additive. (iii) The most compliant element stores the major portion of the elastic deformation energy, and it contributes most to the deformation angle.

**Torsional Compliance of the Rotor Portion in EF\(_1\).** The evaluation of the original data shown in Fig. 1C according to Eq. 2 yielded a stiffness of \( \approx 1,500 \text{pNnm} \) for the mutant βD380CγA87C, where the disulfide link was placed close to the bead (green) and 500 pNnm for the mutant αE284CyA270C (magenta), where it was farther down. The greater figure in the former mutant comprised compliance from all elements of the surface-to-enzyme-to-bead construct, in particular the rotational compliance of the His tags, and of the bead’s attachment to the central shaft. All these “background compliances” were small, and the enzyme body and its attachment to the surface were stiff. The great stiffness was partially relieved in the mutant where the rotor/stator lock was placed farther down toward the C terminus of subunit γ (Fig. 1C, magenta). The increase of the compliance was thus attributable to the portion of subunit γ lying between the 2 sites chosen for blocking the rotation. We calculated a stiffness of \( \kappa = 750 \text{pNnm} \) for the particular portion of the central stalk lying between the green- and magenta-encircled sites as shown in Fig. 1C.
tional freedom of the bead in response to the magnetic field was limited. For both directions of rotation, the probability distribution showed 2 peaks. The respective forward directed peak was narrower and taller than the one where the enzyme had snapped back after being pressed to its forward limiting position. When the magnetic field was off, the bead fluctuated approximately around the middle position of its forced rotation. If, in some single molecules, this approximate symmetry was absent (dominating surface contacts?) the respective data were discarded. The reproducibility of the thermal fluctuations after switching off the magnetic field after a counterclockwise and a clockwise turn is demonstrated by the red and the blue histograms in Fig. 4

**Summary and Discussion**

Fig. 5 summarizes the data. It shows a section through the enzyme in gray and, in color, the cysteine pairs serving to establish a disulfide lock between rotor and stator. The total stiffness, \( k_{\text{total}} \), as detected when closing the respective disulfide cross-link (its 2 cysteines shown in the same hue, dark on the stator or light on the rotor) was closed. The numbers between the black vertical arrows denote the stiffnesses of the rotor domain lying between the respective pairs of cross-link positions. The red arrow marks the region of greatest compliance in \( \text{EF}_0\text{F}_1 \), the dominant elastic buffer that is responsible for an elastic power transmission between \( \text{F}_0 \) and \( \text{F}_1 \).

**Fig. 4.** Immobilized \( \text{EF}_0\text{F}_1 \) with a Q-dot-doped magnetic bead attached to the C-terminal end of both copies of subunit b (A) and histograms of rotary fluctuations (B) with the magnetic field off (Upper) and slowly (0.125 revolutions per second) rotating (Lower). The rotation was either clockwise (when viewed from the \( \text{F}_0 \) side), shown in blue, or counterclockwise, shown in red. The fluctuations in the absence of the magnetic field (Upper) shown in blue and red, respectively, were observed subsequent to a previous clockwise and counterclockwise motion. The points are experimental, and the lines fits with a single Gaussian (Upper) and with 2 Gaussians each (Lower).
mechanical engineering would have placed it; and (v) if the DELSEED lever is free to undergo hinge motion, as during the ATP-waiting dwell, it contributes another rotary torsional compliance with a stiffness of $\approx 50$ pNnm.

Hydrolysis of ATP by EF$_{1}$F$_{1}$ produces an average torque of 50 pNnm as determined from the curvature of a F$_{0}$-attached actin filament by the same single-molecule setup illustrated in Fig. 2A (4). If the holoenzyme operates in its native coupling membrane, and if it reaches thermodynamic equilibrium, then the forward torque generated by ionmotive force (for F$_{0}$) counterbalances the backward torque by ATP hydrolysis (by F$_{1}$), and the elastic element between them is maximally wound up. Taking the above-determined stiffness of the most-compliant domain of EF$_{1}$F$_{1}$, as 68 pNnm, it implies that the rotor is twisted by an angle of $\phi = M/\kappa = 2$ or 4$^\circ$, whereas the eccentric bearing is only negligibly twisted by $<2^\circ$. This amounts to the storage of elastic energy of $U = MF/2\kappa = 18$ pNnm or 11.1 kJ/mol.

How do these figures compare with those that have been hypothesized or indirectly inferred in previous studies? Simulations of the kinetic behavior of the enzyme taking the transient storage of elastic energy have led to gross estimates for the torsional stiffness of the elastic buffer, 60 pNnm (10) and 30 pNnm (14), respectively. A stored elastic energy (6 kBT $\rightarrow 24.7$ pNnm $\rightarrow 14.9$ kJ/mol) has been calculated for the elastic hinge motion in subunit $\beta$ (15). The former figures resulted from rather indirect kinetic or theoretical approaches. The experimentally observed smoothing of the discrete power strokes of F$_{1}$ after being transmitted to F$_{0}$ has led to an estimate for stiffness in the order of 60 pNnm (4). The figures for the stiffness of the major elastic element between F$_{0}$ and F$_{1}$ resulting from the present work, $\kappa = 68$ pNnm, and for the buffered elastic energy, $U = 11$ kJ/mol, are of the same magnitude as the former ones. However, the elastic buffer has now been experimentally attributed to a given domain, namely the bulky segment including the large, globular domains of subunits $\gamma$ and $\epsilon$ plus the loops of the ring of subunit $\epsilon$ to which they are attached.

Our results qualified the role of 3 enzyme domains that were tentatively discussed in this role: (i) The eccentric bearing was rather stiff (see Fig. 4B) for the large binding strength of subunits $\delta$ and $\beta_{2}$ to the hexagon of $(\alpha_{0})_{6}$, see refs. 22–24, and for a comprehensive review on $\delta$ see ref. 21). (ii) The elasticity of the hinge motion of the DELSEED region of subunit $\beta$ has been previously emphasized in theoretical studies (15, 25). We have found that it matters during the turnover of the active enzyme, but it is not the main determinant of the elastic power transmission. (iii) When discussing the inner elasticity of F$_{1}$F$_{1}$, several groups (e.g., ref. 26) including our own have emphasized a role of the coiled coil segment including the large, globular domains of subunits $\gamma$ and $\epsilon$ plus the loops of the ring of subunit $\epsilon$ to which they are attached.

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Preparation of EF$_{1}$ and EF$_{0}$. The preparation of EF$_{1}$, derived from the plasmids pSW3 and pMM25, followed the same procedures published previously (27, 42). EF$_{1}$F$_{1}$, derived from the plasmids pGH14, pGH33, and pGH47, was prepared and purified via its Strep-tags as described in ref. 16.

EF$_{0}$. Based on pSD308, lacking the Strep-tag in c, was purified via its His tags as follows: Membrane protein was obtained as above, followed by centrifugation at 100,000 $\times$ $g$ for 90 min. The supernatant was diluted with buffer A [20 mM Tris-HCl (pH 7.5), 30 mM KC1, 5 mM MgCl$_{2}$, 10% (vol/vol) glycerol, 20 mM imidazole, 0.5% (w/v) N-acetyl-o-glucoamylase final concentration and applied (1 L) to an empty NAP$^{+}$ column packed with 1 ml of Ni$^{2+}$-NTA Superflow. Wash was done with 5 ml of buffer A and then with the same buffer containing 150 mM imidazole. Glyceral (70%) was added to eluates before they were quick-frozen in liquid nitrogen and stored at $\approx$ –80°C before use.
Preparation of F-Actin. The preparation of F-actin followed the same procedures published previously (16).

Preparation of Magnetic Beads. Streptavidin-coated hyperparamagnetic beads (stock solution 10 mg/ml, diameter 1 µm; Roche) for experiments with EF1 or EFOF1 were diluted 10-fold with 50 mM Mops/KOH (pH 7.5), 50 mM KCl, and 5 mM MgCl2 (buffer B) or 50 mM Mops/KOH (pH 7.5), 50 mM KCl, 5 mM MgCl2, 0.5% (v/v) N-octyl-β-D-glucopyranoside, and 10% (v/v) glycerol (buffer C), respectively. The dilute suspension was centrifuged (16,000 × g, 4 °C, 3 min), and the pellet was resuspended in buffer B or buffer C. This washing procedure was repeated 3 times.

Immobilization of EF1 and EFOF1. Similar procedures were used for immobilizing EF1 and EFOF1. Samples were filled into flow cells consisting of 2 coverslips (bottom 26 × 76 mm²; top 24 × 24 mm²) separated by double-adhesive tape (Tesa). EF1/EFOF1 protein solutions were stepwise infused as described previously (16) (using buffer C in all steps). 50 µg-M–5 mM ATP was used in the last step. For ADP inhibition, 50 µl of 20 mM glucose, 0.2 mg/ml glucose oxidase, 50 µg/ml catalase (oxygen scavenger system, OSS), 0.5% 2-mercaptoethanol (2-mer), 1 unit/ml hexokinase, and 5 mM ADP in buffer C were added after washing with 50 µl of buffer C.

The infusion order for EF1 was as follows (50 µl per step, 4-min incubation, wash with buffer B before each step): (i) 0.8 µg Ni-NTA-agarose peroxidase conjugate in buffer B; (ii) 10 mg/ml bovine serum albumin in buffer B; (iii) 5 µM EF1; in buffer B; (iv) 0.2 mg/ml magnetic beads in buffer B (7-min incubation); (v) 2 PM Q-dots (stock 2 µM, dilution factor 1:1.000.000; Quantum Dot) in buffer B (7-min incubation); (vi) OSS, 0.5% 2-mer, 20 mM DTT, and 5 mM ATP in buffer B.

For psd308-based EF1/Fo, the incubation followed the same instructions as for EF1 (without 20 mM DTT in step vi), but replacing buffer B with buffer C.

For oxidation of both EF1/Fo and EF1, 50 µl of OSS, 0.2 mg/ml creatine kinase, 2.5 mM creatine phosphate, 1–2 mM Ellman’s reagent, and 5 mM ATP in buffer C or buffer B were added after washing with 50 µl of buffer C or buffer B, respectively. For reducing EF1, after oxidation, 50 µl of OSS, 0.5% 2-mer, 20 mM DTT, 10 mM Na2SO3, and 5 mM ATP in buffer B were added after washing with 50 µl of buffer B.

Video Microscopy. EF1 and EFOF1 constructs were observed, and single-molecule rotation was recorded with an inverted fluorescence microscope as published (16). Fast bleaching of the fluorescently labeled actin filaments in the oxidized state was overcome by reducing the excitation intensity. Video data were captured with a Pinnacle DV500 Plus video card, and digitized with Adobe Premiere 6.0. A software program for evaluation of the obtained video sequences was written with Matlab 7.

Homology Modeling. Our model of EF1/Fo was based on the structure as determined by Abrahams et al. [PDB ID Code 1bmf, (6)]. Modeling was carried out with the programs Whatfit (44) and O (45). The model coordinates are available from www.biologie.uni-osnabrueck.de/Biophysik/Engelbrecht/ef1/data/ef1.

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