Protonation-dependent stepped rotation of the F-type ATP synthase c-ring observed by single-molecule measurements

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The two opposed rotary molecular motors of the F₀F₁-ATP synthase work together to provide the majority of ATP in biological organisms. Rotation occurs in 120° power strokes separated by dwells when F₁ synthesizes or hydrolyzes ATP. F₀ and F₁ complexes connect via a central rotor stalk and a peripheral stator stalk. A major unresolved question is the mechanism in which the interaction between subunit-a and rotating subunit-c–ring in the F₀ motor uses the flux of H⁺ across the membrane to induce clockwise rotation against the force of counterclockwise rotation driven by the F₁-ATPase. In single-molecule measurements of F₀F₁ embedded in bilipid nanodiscs, we observed that the ability of the F₀ motor to form transient dwells increases with decreasing pH. Transient dwells can halt counterclockwise rotation powered by the F₁-ATPase in steps equivalent to the rotation of single c-subunits in the c–ring of F₀, and can push the common axle shared by the two motors clockwise by as much as one c-subunit. Because the F₀ proton half-channels that access the periplasm and the cytoplasm are exposed to the same pH, these data are consistent with the conclusion that the periplasmic half-channel is more easily protonated in a manner that halts ATPase-driven rotation by blocking ATPase-dependent proton pumping. The fit of transient dwell occurrence to the sum of three Gaussian curves suggests that the asymmetry of the three ATPase–dependent 120° power strokes imposed by the relative positions of the central and peripheral stalks affects c-subunit stepping efficiency.

The F-ATP synthases, located in eubacteria, mitochondria, and chloroplasts, are members of a family of rotary molecular motors that also include archaeal A-ATP synthases, bacterial A/V-like ATP synthases, and eukaryotic vacuolar V-ATPases (1). The F-ATP synthases are composed of two opposed rotary motors known as the membrane-embedded F₁ and the extrinsic membrane protein complex F₀ (Fig. 1). These motors are connected by a central stalk that serves as the rotor and a peripheral stalk that is a component of the stator (2). In Escherichia coli, as in most organisms, the largest single source of cellular ATP is that made by the F₀F₁-ATP synthase.

Synthesis of ATP from ADP and inorganic phosphate (Pₐ) is catalyzed by F₁ in response to a transmembrane electrochemical gradient used by F₀ to force the rotor in the clockwise (CW) direction as viewed from the periplasm of E. coli (3). In so doing, E. coli F₀, which is specific for protons, consumes the gradient by transporting protons across the membrane. The F₁ motor can hydrolyze ATP to drive the rotor counterclockwise (CCW) and pump protons (4, 5). However, under steady state conditions, the F₀F₁-ATP synthase maintains the [ATP]/[ADP] [Pi] ratio far from equilibrium (6–8).

The three catalytic sites for ATP synthesis/hydrolysis are located at the interfaces of α- and β-subunit heterodimers that comprise the (αβ)₃-ring of the F₁-ATPase, which serves as the stator (9). The rotor consists of the γ-subunit that protrudes from the center of the (αβ)₃-ring, the ε-subunit, and the ring of c-subunits of F₀. During ATPase-driven rotation, each 120° power stroke occurs as the result of the binding of an ATP to the empty catalytic site, whereas product release occurs at an adjacent site (10). Hydrolysis of ATP occurs at the third site during catalytic dwells that separate the power strokes. Three states of the E. coli F₀F₁ structure were revealed by single-particle cryo-EM in which the axle subunits γ and ε were rotated in 120° increments from the peripheral stalk that is a component of the stator (11).

In the F₀ motor, proton translocation-dependent rotation of the c–ring results from events in which half-channels in stator subunit-a protonate and deprotonate cAsp⁶¹ on each c-subunit. Subunit-a of E. coli F₁Fₐ folds in a manner similar to that observed in F₀ structures from other organisms for which the structure is known (11–14). Subunit-a helices 1–4 anchor the b-subunits of the peripheral stalk, and are distal from the c–ring. Helices 3–6 are oriented at an oblique angle to the membrane plane where C-terminal helices (5 and 6) are proximal to the c–ring. The connection between the distal and proximal helices contains two 3₁₀-helical segments (Fig. 1, B and C, helices I and II) that vary in conformation among known structures.

Subunit-a residues aAsn²¹⁴, aGlu²¹⁹, aHis²⁴⁵, and aGln²⁵² on helices 5 and 6 have been implicated in proton translocation as part of the periplasmic half-channel that serves as the import channel during proton gradient–powered c–ring rotation (CW) for ATP synthesis (15–17). To date, the only residue known to participate in the cytoplasmic half-channel, which serves as the import channel for ATPase-driven proton pumping, is aGlu¹⁹⁶ (17, 18). The two half-channels are separated by aArg²¹⁰ that...
effectively deprotonates cAsp61 as each successive c-subunit rotates into close proximity of its resonance-stabilized positive charge (19).

Rates of ATP synthesis catalyzed by the F-ATP synthase can approach 400–600 ATP s⁻¹ (20–22). This equates to 500–750 s⁻¹ per proton translocated during the rotational stepping of single c-subunits in E. coli that has a c₁₀-ring. The ability to observe single c-subunit stepping of the c₁₀-ring on this time scale was first achieved in single-molecule experiments by monitoring ATPase-driven rotation using a gold nanorod attached to the c-ring of F₀F₁ embedded in lipid bilayer nanodiscs (n-F₀F₁) (23). Although some power strokes rotated CCW in continuous 120° events, many were periodically interrupted by “transient dwells.” These dwells occurred at an average interval of ~36°, consistent with single c-subunit stepping of the c₁₀-ring of E. coli F₀F₁. The duration of transient dwells ranged from 50 to 175 s. Although somewhat shorter than the duration of c-subunit stepping anticipated during ATP synthesis, transient dwells are observed in the presence of high ATP concentrations that optimize ATP hydrolysis in lieu of synthesis. The fraction of power strokes that contain transient dwells can be increased to the point at which they are present in all power strokes of most data sets (~80% average occurrence in all power strokes) through the use of viscosity-dependent drag to decrease the angular velocity of the F₁-ATPase power stroke.

Mutations in subunit-a and subunit-c have been identified that affect the ability to form transient dwells (18, 23), indicating that these dwells result when subunit-a forms a “leash” with the c-ring that limits CCW rotation to a maximum of ~36° while engaged. Subunit-a was not only observed to halt CCW rotation of the c-ring in n-F₀F₁ but, in >70% of all transient dwells, was also able to push the rotor CW against ATPase-dependent rotation powered by F₁ (18). The extent of CW rotation in the ATP synthesis direction averaged 11°, but could be as much as ~36°, which is equivalent to rotation of the c-ring by one c-subunit relative to subunit-a.

Using proteoliposomes in which the F₁ complex of F₀F₁ is on the outside, high rates of ATP synthesis require transmembrane proton gradients with a ΔpH = 3. This is typically achieved by equilibrating the pH inside the liposome to 5.5 and then rapidly shifting the outside pH to 8.5 (6, 21). Thus far, single-molecule rotation studies of F₀F₁ embedded in nanodiscs have been examined only at pH 8.0. We now present a single-molecule study of the pH dependence of transient dwell formation by n-F₀F₁. The occurrence of transient dwells increased inversely with pH over the pH range of 5.0 to 7.0. Transient dwells formed at low pH at intervals of ~36° lasted an average of 118–158 s and were able to push the c-ring CW against the force of ATPase-dependent CCW rotation by as much as one c-subunit. These results show that even though both F₀ half-channels were exposed to the same pH, the periplasmic half-channel that serves as the ATP synthase proton import channel is more easily protonated in a manner that halted ATPase-driven rotation by blocking ATPase-dependent proton pumping.

Results

Rotation of the c-ring of single n-F₀F₁ molecules was monitored as a function of time by changes in the intensity of polarized red light scattered from a 80 × 40-nm gold nanorod attached to the c-ring (Fig. 2). For each molecule examined, multiple 5-s data sets of ATP hydrolysis-powered rotation were acquired in the presence of 1 mM Mg-ATP at a data acquisition rate of 100 kHz. At this saturating substrate concentration, E. coli F₁ produces power strokes that rotate 120° CCW on a time scale of ~300 μs, which are separated by catalytic dwells with a duration of a few milliseconds (5, 24). The intensity of polarized red light that is scattered from the long axis of the
**Figure 2. A gold nanorod was used as a probe of rotational position to make single-molecule measurements of F₀F₁, embedded in lipid bilayer nanodiscs (green circle).** Attachment of F₀F₁ to the microscope slide occurred via His₆ tags on the N termini of the F₁ β-subunits. The c27Cys mutation to E. coli F₀F₁ (see "Experimental procedures") was biotinylated to attach the gold nanorod (80 × 40 nm) coated with streptavidin (blue sphere). The 120° power strokes (red and green dashed arrows) resulting from F₁-ATPase–dependent CCW rotation in the presence of 1 mM Mg-ATP were monitored by the intensity of polarized red light scattered from the long axis of the nanorod as a function of time. Shorter wavelengths were eliminated by a band-pass filter. Prior to data collection, a polarizing filter (white arrow) was aligned for minimum intensity of scattered light at one of the three ATPase–dependent catalytic dwells (0°) such that the intensity during the subsequent power stroke increased and passed through a maximum when the nanorod rotated 90° measured using an avalanche photodiode. These power strokes (red dashed arrow) were analyzed for the presence of transient dwells.

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**pH dependence of rotational F₀F₁ c-ring stepping**

Fig. 5 shows the average percent of transient dwells formed per data set during n-F₀F₁ power strokes as a function of pH, where each data set contained about 300 power strokes. At pH 9.0, about 23% of the power strokes contained transient dwells, and transient dwell formation did not change significantly from this value between pH 9.0 and pH 7.5. However, the occurrence of transient dwells increased with decreasing pH between pH 7.5 and pH 5.0 to an average of ~48% at pH 5.0. The fit of these data to the Henderson-Hasselbach relationship suggested that protonation of a group with a pKₐ of 6.3 was necessary to form a transient dwell. However, it is clear that the average changes in transient dwell formation between pH 6.0 and 7.5 did not fit particularly well to the dependence on the protonation of a single group.

The distributions of single-molecule rotation data sets of n-F₀F₁ power strokes at each pH as a function of the percentage of transitions in each data set that contain transient dwells are shown in Fig. 6A (gray bars). The percentage of transient dwells formed per data set that each contained ~300 power strokes was determined, and the data sets were binned into groups for each 10% occurrence of transient dwells formed. Although the percentage of transient dwells formed per data set shown in Fig. 5 was taken from the averages of these distributions, it is clear that these data did not fit to a normal distribution over the pH range examined. In several data sets at pH values between 5.0 and 6.0, transient dwells formed in 90–100% of the power strokes.

The E. coli F₀F₁ structure determined by cryo-EM was observed to exist in three states that differed in the rotary positions of the central stalk relative to the peripheral stalk by 120° (11). To test the hypothesis that the distributions of transient dwells observed in Fig. 6A could result in differences of the three structural states, three Gaussian curves for which the probability of forming transient dwells was low (blue line), medium (red line), and high (green line) were generated. The sum of the three Gaussians (black line) were fit to the measured distribution at each pH (gray bars).

The average values of transient dwells formed represented by each of the three Gaussian curves increased inversely with pH (Fig. 7). The Gaussian fit with the highest probability of forming transient dwells (green) increased to the greatest extent, and had an average value of 70% transient dwell formation per data set at pH 5.0.

The distributions of the extent to which subunit-α was able to force the c-ring to rotate CW against the ATPase-driven CCW power stroke during the transient dwell as a function of pH are shown in Fig. 6B. The maximal extent of these "backsteps" in the CW direction was about 36°, equivalent to the rotation of the c₁₀-ring by one c-subunit. The distributions of the extent of the backsteps was plotted as a difference from that of pH 5.5 for which the extent of the backstep was lowest (Fig. 6C). The average changes in the extent of the backstep as a function of pH were insignificant, ranging from ~11° at pH 5.5 to a maximum of about 13° at pH 7.0. The percentage of transient dwells that contained backsteps varied only by ~13% as a function of pH from a minimum to a maximum of 67 to 80% at pH 5.0 and 7.0, respectively (Fig. 8).
The results presented here show that the occurrence of transient dwells increases as the pH of the medium becomes more acidic. Subunit-a is the stator component in the F0 complex that contains both half-channels for proton translocation across the membrane (27). High rates of ATP synthesis are achieved in inverted membrane vesicles of *E. coli* when the periplasmic and cytoplasmic half-channels are exposed to pH 5.5 and 8.5, respectively (6, 21). As a result of this non-equilibrium gradient, protons enter F0 through the periplasmic half-channel (the ATP synthase input channel) to induce CW rotation of the c-ring. Conversely, ATPase-driven proton pumping occurs as the result of protons entering F0 from the cytoplasm as the result of CCW rotation induced by ATP hydrolysis in F1.

Transient dwells result from the ability of subunit-a to interact with subunit-c in a manner that stops ATPase-dependent CCW rotation of the c-ring, and results in CW rotation in the ATP synthesis direction ~ 80% of the time (Fig. 8). Modification of cAsp61 in the c-ring by N,N'-dicyclohexylcarbodiimide (DCCD) effectively inhibits ATPase activity of F0F1 embedded in nanodiscs (23), indicating that this ATPase-dependent rotation is linked to proton pumping across the lipid bilayer.

A major difference between a membrane and a nanodisc is that in the latter, which was used in the single-molecule experiments here, both half-channels are exposed to the same pH. The rate of F1-ATPase activity measured in ensemble assays decreases with pH between pH 7.5 and 6.5 by ~2-fold (28), during which the increase in transient dwells is the greatest (Fig. 5). However, it is noteworthy that the rate-limiting step that determines ATPase activity occurs during the catalytic dwell, and not angular velocity of the power stroke (26), which can contribute to the formation of transient dwells (23). Thus, although the potential contribution of the pH dependence to the angular velocity of the F1-ATPase cannot be ruled out, these data strongly suggest that the periplasmic half-channel is more easily protonated than is the cytoplasmic half-channel.

This preferential protonation of the periplasmic half-channel halts ATPase-driven rotation, and thereby blocks ATPase-
dependent proton pumping. Residues that contribute to proton translocation include Asn\textsuperscript{214}, Glu\textsuperscript{219}, His\textsuperscript{245}, and Gln\textsuperscript{252} in the periplasmic half-channel, and Glu\textsuperscript{196} in the cytoplasmic half-channel (15–18). Several additional residues are likely to have a role, especially in the cytoplasmic half-channel that is currently less well defined.

When ATP synthase is driven by a high non-equilibrium pH gradient (\(\Delta p\text{H} = 3\)), the periplasmic half-channel is exposed to a proton concentration 1000-fold higher than is the cytoplasmic channel. At such a high concentration, the groups in the former half-channel will be rapidly reproto-
nated after each donation of a proton to cAsp\textsuperscript{61}, which induces CW rotation of the c-ring, and minimizes the prob-
ability that the c-ring will subsequently rotate CCW. In response to CW rotation, a protonated cAsp\textsuperscript{61} moves into

![Figure 6. The pH dependence is shown. A, the distribution of single-molecule n-F\textsubscript{0}F\textsubscript{1} power stroke data sets as a function of the percentage of the occurrence of transient dwells per data set, which were binned to each 10\% (gray bar graphs). Each data set contained ~300 power strokes. The fit of the data to the sum of three Gaussians (black line) where the probability of forming transient dwells was low (blue line), medium (red line), and high (green line). The total number of n-F\textsubscript{0}F\textsubscript{1} molecules examined at each pH is indicated. B, the distribution of the extent of F\textsubscript{0}-dependent CW rotation against the force of F\textsubscript{1}-ATPase–driven rotation. C, the difference in extent of CW rotation at each pH versus that observed at pH 5.5.](image-url)
this half-channel such that dissociation of the proton into the cytoplasm is favored.

The concentration of protons required to protonate each half-channel is likely to depend on the number of groups that can be protonated, their aggregate accessibility to the adjacent aqueous phase, and their pKs, which depend on the hydrophobicity of their environments. The results presented here show that the ability to protonate the half-channels differs. Mutational studies are required to determine the relative contributions of subunit-a residues. These differences in the two half-channels have likely evolved to favor proton flux in the ATP synthase direction to minimize the futile cycle when the system has reached a steady state chemical gradient of ATP to ADP and P_i. In such a futile cycle, an ATP synthesized by proton flow would be immediately hydrolyzed to pump the protons in the opposite direction with no net gain in the storage of energy in the form of ATP. Without a bias that favors protonation of the ATP synthase half-channel, the magnitude of the chemical gradient that can be sustained will be diminished.

Single particle cryo-EM structures of F_oF_1 in the autoinhibited form (11) revealed three states (Fig. 9). The autoinhibited form of the ATP synthase from *E. coli* results when the C-terminal helix-turn-helix domain of the ε-subunit has become inserted into the core of the (αβ)_3-ring along the shaft of the γ-subunit in a manner that prevents rotation (29). The three structural states of this F_oF_1 structure are distinguished by the rotary positions of central stalk subunits γ and ε relative to the peripheral stalk that are separated by 120°, which is equivalent to the rotary positions of the drive shaft between ATPase-driven power strokes. It is noteworthy that single-molecule FRET measurements made where the donor and acceptor are attached to the central and peripheral stalks show a sequential low, medium, and high FRET signal when F_oF_1 embedded in proteoliposomes are actively synthesizing ATP in response to a proton gradient (3). The correlation between these FRET signals with the relative positions of the central and peripheral stalks in the three states of the *E. coli* cryo-EM structure suggests that the rotary positions of the γ and ε-subunits in these three states resemble that of the functional protein complex during each of the three F_oF_1-catalytic catalytic dwells.

The data presented at each pH in Fig. 6A represent the sum of data sets typically from ~50 molecules of F_oF_1. The occurrence of transient dwells was analyzed for each molecule examined only from the power stroke in which the scattered light intensity of the nanorod progressed from a minimum through a maximum (Fig. 2), and not from the other two power strokes that complete a 360° rotation. For any given molecule, there is an equal probability that the power stroke analyzed corresponds to the rotation from one of the three structural states defined by the relative position of the two stalks. Thus, for every 51 F_oF_1 molecules analyzed for transient dwells, it is likely that the power strokes specific to each of the three different structural states will have been sampled by 17 molecules. This conclusion is supported by the observations that the distributions of transient dwell occurrence at any of the pH values examined do not fit to a single normal distribution, but that good fits to the data were achieved by fitting the distributions to the sum of three Gaussians (Fig. 6A).

The average values derived from each Gaussian dependence changed by a comparable extent as a function of pH (Fig. 7), which implies that the Gaussian curves represent molecules in which the probability of forming a transient dwell is low (blue), medium (red), or high (green). This suggests that the ATPase-driven power stroke that follows each of the three structural F_oF_1 states derived from cryo-EM has a low, medium, or high probability of forming transient dwells. In the absence of knowledge of the correlation between the ability to form transient dwells and the structural state of the power stroke examined, it is not possible at this time to know how the probability to form transient dwells in any one structural state changes with pH. It is noteworthy that because the ATPase rotates in 120° power strokes, the c-ring will rotate 4 × 36° for one of the power strokes, but 3 × 36° for the other two. Thus, it is possible that the probability of observing transient dwells is more likely for molecules aligned to step by four c-subunits per power stroke, although it does not explain the differences in the molecules with a low and medium probability of forming transient dwells.

The three states of *E. coli* F_oF_1 determined by cryo-EM (Fig. 9) differ not only by the relative position of the central and peripheral stalks, but also by the conformation of subunit-a, and its position on the c-ring. These differences include the positions of subunit-a residues Asn_214, Arg_210, and Glu_196 involved in proton translocation relative to the cAsp_61 residues on each c-subunit. In addition, the three structural states of *E. coli* F_oF_1 differ in the position of the subunit-a 3_10-helix II and...
its subsequent loop relative to the c-ring. It is noteworthy that similar conformational changes were proposed in the grab and push mechanism to explain the basis of transient dwell formation, and the ability of subunit-a to push the c-ring CW against the force of ATPase-dependent rotation (18). The cryo-EM structures of *E. coli* F$_0$F$_1$ are in the autoinhibited state in which the C terminus of the e-subunit has adopted a conformation that prevents rotation. Thus, it is not possible at this time to determine whether subunit-a has been trapped in different functional conformations that represent sequential steps in the grab and push mechanism or whether these conformational differences predispose a given rotational state of the complex to have a low, medium, or high probability of forming transient dwells.

**Experimental procedures**

**Preparations of n-F$_0$F$_1$ ATPase**

Detergent-solubilized F$_0$F$_1$ samples were expressed from the pNY1-Ase plasmid construct that contained a Cys inserted at the second position of subunit-c (c2/Cys) as described previously by Ishmukhametov et al. (23). The protein complex was purified, biotinylated, and incorporated into nanodiscs as described previously (23).

**Single-molecule studies**

Assembly of n-F$_0$F$_1$ complexes on a microscope slide and attachment of a 80 x 40-nm gold nanorod to the c-ring of each complex was carried out as described by Martin et al. (18). Rotation measurements were made in buffer containing 1 mM Mg$^{2+}$ ATP, 10 mM KCl, 30 mM PIPES, and 30 mM Tris that was adjusted to the pH indicated. The assay to use changes in scattered light intensity from the nanorod to measure rotation of single molecules was as described by Hornung et al. (25). Briefly, light scattered from a single nanorod on the microscope slide (as shown in Fig. 2) was aligned with a pinhole, which then passed through a band-pass filter to eliminate all but red light, a polarizing filter, and a lens that focused the light on a single photon counter. Prior to data acquisition, the polarizing filter was rotated and light intensity changes were monitored at a frame rate equivalent to ~55 frames per second. At this slow frame rate, the observed changes in light intensity primarily represented the three rotational positions of the nanorod defined by the catalytic dwells of the F$_1$-ATPase that are separated by 120°. Attachment of the nanorod to an actively rotating n-F$_0$F$_1$ complex was confirmed by the change in the dynamic range of the changes in scattered light intensity as a function of the rotational position of the polarizing filter as described by Spetzler et al. (24). The filter was then rotated to align one of the catalytic dwells with a minimum of scattered light intensity. Rotation data were then acquired at 100 kHz in 5-s data sets. Power strokes that rotated the nanorod from a minimum through a maximum in scattered light intensity, which is equivalent to a 90° transition in position, were analyzed for the presence of transient dwells using custom software developed in MATLAB R2013b as described by Martin et al. (18).

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