Comparison between single-molecule and X-ray crystallography data on yeast F1-ATPase

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Single molecule studies in recent decades have elucidated the full chemo-mechanical cycle of F1-ATPase, mostly based on F1 from thermophilic bacteria. In contrast, high-resolution crystal structures are only available for mitochondrial F1. Here we present high resolution single molecule rotational data on F1 from Saccharomyces cerevisiae, obtained using new high throughput detection and analysis tools. Rotational data are presented for the wild type mitochondrial enzyme, a “liver” isoform, and six mutant forms of yeast F1 that have previously been demonstrated to be less efficient or partially uncoupled. The wild-type and “liver” isoforms show the same qualitative features as F1 from Escherichia coli and thermophilic bacteria. The analysis of the mutant forms revealed a delay at the catalytic dwell and associated decrease in Vmax, with magnitudes consistent with the level of disruption seen in the crystal structures. At least one of the mutant forms shows a previously un-observed dwell at the ATP binding angle, potentially attributable to slowed release of ADP. We discuss the correlation between crystal structures and single molecule results.

F1

F1-ATP synthase is a rotary molecular complex responsible for the synthesis of ATP in bacteria and eukaryotes under aerobic conditions. ATP synthase couples energy from the electrochemical gradient across the cytoplasmic, mitochondrial, or chloroplast membrane, to the phosphorylation of ADP to ATP. The enzyme is comprised of two separable coaxial motors: F1 and Fo. F1 is water-soluble and contains the catalytic sites used for the synthesis of ATP. Fo is membrane bound and acts as a proton turbine, using the electrochemical gradient to generate rotation that is physically coupled to F1 rotation. When isolated from Fo, F1 behaves as an ATP-hydrolysing enzyme.

Figure 1A shows the atomic structure of yeast F1, and Figure 1B the mechanochemical cycle that couples ATP hydrolysis to rotation of the γ subunit in F1 from the thermophilic Bacillus PS3 (TF1)1 (for reviews, see Refs. 2–5). This enzyme’s high functional stability and slow kinetics at room temperature facilitate extended single-molecule observation and resolution of mechano-chemical transitions. F1 from E. coli rotates 4–5 times faster at room temperature, with a similar mechanism6.

The TP, E and DP sites of the ground state crystal structure are believed to correspond to the states of subunits β,–β, respectively at the 80° catalytic dwell, as illustrated in Fig. 1B9,10. All crystal structures to date have γ subunits within 20° of the ground state angle, most within 5°, indicating that all are variations on this ground state10. About half of these structures contain phosphate or an analogue at the E site, consistent with phosphate release at 80°. TP, E and DP states are best defined by their position with respect to the asymmetric γ subunit, as their nucleotide occupancy varies across different crystal structures. We label sites at the 0° dwell E*, TP*, and DP*, corresponding to the ground state they were most recently in if rotating in the ATP hydrolysis direction. A mechanochemical cycle can then be defined whereby a single β subunit in the E* conformation binds ATP at 0°, then moves through the TP and TP* conformations to hydrolyse ATP in the DP conformation at 200° and release ADP at 240° (DP*). Following rotation to 320° (E) it releases phosphate and is ready to bind ATP again at 360°11. The other two β subunits follow the same cycle delayed by 120° and 240°. TF1 and EF1 rotate unidirectionally but not continuously, stochastically entering a paused state due to Mg2+13. ADP inhibition15.

Importantly, while most single molecule studies have been performed in TF1, the majority of high resolution crystal structures are for mitochondrial F1, with high resolution structures of bacterial F1 proving much harder to obtain14,15. Recent single-molecule studies of EF14 have demonstrated the ability to resolve the kinetics of mesophilic
F₁ at room temperature. A very recent study of human F₁ (HF₁) reported that phosphate release and hydrolysis occur at 65° and 90° respectively after the previous ATP binding dwell, rather than both at 80° as in TF₁ and EF₁. Single-molecule experiments on F₁ from yeast will allow us to compare the mechanochemical cycles of different mitochondrial enzymes and to determine to what extent the extensive knowledge of TF₁ can be applied to mitochondrial F₁. It also allows the first comparison between single-molecule rotation studies and multiple high resolution crystal structures of F₁ from the same organism. High-resolution X-ray crystal structures have been determined for bovine F₁ in a number of different states, including a transition state with AlF₄:ADP⁻⁻⁻⁺ and for wild type yeast F₁ in both the presence and absence of bound nucleotides. For the yeast enzyme, the crystal lattice contained 3 complexes, with a well resolved phosphate bound to the E site in Complex II but absent in Complex I and III.

In this paper, we investigate two classes of mutations to yeast F₁: mitochondrial genome integrity mutations and the “liver” isoform. Mitochondrial genome integrity (mgi) is a class of mutations in the genes encoding the α, β, and γ subunits that allow petite negative yeast K. lactis to lose mitochondrial DNA. While these mutations have been mapped exclusively to the α, β, and γ subunits of ATP synthase (Fig. 1A), the relationship between their effect on ATP synthase and the resulting yeast phenotypes is unclear. X-ray crystal structures have been determined for four mgi mutations. These structures suggest two mechanisms for uncoupling and identify crucial regions involved. Mutations were grouped into two classes according to which mechanism is postulated: Class 1 mutations alter the structure of the E site and apparently disrupt phosphate binding, while Class 2 may disrupt interaction of the central stalk with the γβ pair.

There are two isoforms of the γ subunit in humans and other mammals: a heart form and a liver form. The heart form is expressed in heart, skeletal muscle, and intercostal muscle diaphragm, tissues of rapid and high-energy demand. The liver form is expressed in liver, cerebellar cortex, cerebrum, thyroid, spleen, pancreas, kidney and testis, tissues of relatively low or steady energy demand. The isoforms are the result of alternative splicing. Isoform switching is regulated in a tissue specific manner and the alternative splicing can be reversibly induced by acid stimulation in muscle cells, but not in non-muscle cells, suggesting that isoforms of the γ subunit in humans are a result of a carefully regulated alternate splicing process. This regulation is relevant to the physiology of the cell, yet the resulting effect on the biochemistry of the ATP synthase is unknown. The γ subunit expressed in the liver differs from that expressed in the heart only by the presence of an additional residue, Asp, at the C-terminus. The tertiary structure of the yeast F₁ is highly conserved with that of the bovine heart F₁. Further, the primary sequence of the α and β subunit of the F₁ ATPase is highly conserved from yeast to human. Based on the crystal structure of yeast and bovine F₁ ATPase, it was predicted that the Asp at the C-terminus of the γ subunit could form a salt bridge with αArg288 in γE (Pagadala V. and Mueller D.M., unpublished results, see SI for further details). If formed, the salt bridge is predicted to slow the catalytic cycle without decreasing the efficiency of the enzyme.

In this study, we use new high throughput detection and analysis tools to investigate the behaviour of wild type mitochondrial F₁ from S. cerevisiae at varying ATP concentrations. We quantify the lifetimes of the ATP-binding and catalytic states, and present evidence that mitochondrial F₁ rotation is governed by the same mechanism as that of the well-studied TF₁ and the more recently studied EF₁. We also use these tools to investigate the behaviour of seven forms of yeast F₁ containing a point mutation, including six mgi mutations and a mutation that mimics the heart-to-liver isoform in humans. For four of these mutants, we discuss structure-function relationships with reference to high-resolution crystal structures.

**Results**

**Yield of rotating gold beads.** The rotation of yeast F₁-ATPase was monitored by darkfield microscopy after attachment of gold particles to the γ subunit, as described in Methods. The position of all gold beads in the field of view was calculated and examined. A small fraction of beads showed clear circular trajectories (Fig. 2A), similar to those presented in previous work on F₁. Many beads rotated unidirectionally as revealed by asymmetry in the power spectrum of their motion, but with signal-to-noise ratios too low for clear circular trajectories (Fig. 2B).

To assess the fraction of beads which were rotating, the directional ‘bias’ in rotation was examined (Methods). Rotation rate was assessed independently by fitting an ellipse to the bead trajectory and tracking the bead’s angular speed around the ellipse - this accurately measures speed in high signal to noise conditions but underestimates speed when noise is significant. Fig. 2D-G shows bias vs. angular speed for F₁-bead complexes at various ATP concentrations under identical imaging conditions. F₁ is not expected to rotate in the absence of ATP, and under such conditions the plot is symmetric around the origin. When ATP is present, a ‘tail’ is visible in the direction corresponding to counter-clockwise rotation. Significantly, there is no corresponding tail in the direction that corresponds to clockwise rotation. This ATP dependent asymmetry indicates that beads with significant values of bias are rotating due to F₁, even though many traces are too noisy to detect clear circular trajectories. In the ‘best samples’, like those shown in Fig. 2, a conservative estimate is that at least 25% of the beads are rotating, and this is discussed further in SI.

The bias of a bead is related to the signal-to-noise value, which suggests that beads with a high bias should have high quality data. Qualitatively, this is observed. In work on bacterial F₁, rotating molecules have typically been identified by eye, a laborious procedure subject to human selection. By measuring the bias of all recorded beads (potentially greater than 1000 per sample), the rotating molecules providing the highest quality data were quickly and automatically identified. In the SI, we discuss why a spread in bias/quality exists, and whether high quality spinners are representative of the broader population. In summary, data quality is principally determined by the orientation of the gold bead bound to F₁, but is unlikely to significantly affect the kinetics. The spread in bias is not unique to F₁ from yeast and is likely a common feature of all F₁ single molecule spinning bead assays.

**Characterisation of wild type F₁.** Fig. 3 shows speeds measured for wild type yeast F₁ as a function of ATP concentration using power spectra, and the more conventional angular rotation speed (Methods). Both fit well to Michaelis-Menten kinetics, with fit parameters of Vₘₐₓ = 586 ± 23 rev/s and Kₘ = 62 ± 9 μM, and 471 ± 79 rev/s and 59 ± 18 μM, respectively. Quoted errors correspond to the 95% confidence range of the fit. The difference between the two fits is primarily a result of selection bias in the smaller number of molecules used for the angular rotation speeds (see SI). Rotation speeds tended to be steady for individual molecules, but varied between molecules at the same ATP concentration, with a spread (sample deviation as fraction of mean) of 16% and 13% for the two methods when averaged over all ATP concentrations. The fit parameters are similar to those determined by biochemical methods (see SI). Biotinylation of the γ subunit caused no significant changes in the steady-state ATPase kinetic values.

Fig. 4B-D shows a single molecule displaying typical stepping patterns for low, intermediate and high ATP concentrations. At low and high ATP concentrations, three dwell positions are observed, while at intermediate ATP concentrations six dwells are visible. This is consistent with the model of F₁ rotation developed using TF₁, in which the ATP binding dwell, observed at low ATP concentrations, is located approximately 40° after the catalytic dwell, observed at high ATP concentrations. By contrast, in HF₁, six...
Figure 1 | X-ray crystal structure and proposed mechanochemical cycle. (A) Locations of the mgi residues in the X-ray crystal structure of yeast F1 (pdb: 2HLD, Complex I, Ref. 22). The αTP, βE, γ, δ, and ε subunits are shown along with the residues that were mutated for this study. Also shown are the Catch 1 and 2 regions which interact with the γ subunit and help define the structure of the catalytic sites. The P-loop, which forms part of the P1 and ATP binding site, is coloured in red and labelled. (B) A proposed mechanochemical coupling scheme of thermophilic F1. The catalytic sites of the three β subunits are shown in orange (β1), green (β2), and purple (β3). We define the 0° binding dwell to correspond to ATP binding at a particular site labelled β1 and ADP release at β2. The subunit shown in orange binds ATP at the 0° mark, and each subunit follows the processes and states listed in the table at the angles shown. The angular position of the γ subunit is represented by the central yellow arrow. Once a catalytic site binds an ATP molecule, it remains bound for 200° rotation of the γ subunit, whereupon it is hydrolysed. ADP is released at 240° and P1 at 320° from the binding site. Each β subunit completes one hydrolysis of an ATP molecule per 360° rotation of the γ subunit, and the three β subunits are staggered in their catalytic state by 120°. This means that at each ATP waiting dwell, ADP will release from one subunit and ATP will bind to the previous subunit. At each catalytic dwell, the ATP bound to one subunit will hydrolyze and the P1 from the previous subunit will be released. Refer to Refs. 2–5 for detailed reviews of the mechanochemical cycle of F1. Fig. redrawn from Ref. 2 with modifications.
dwell positions were reported at high ATP concentrations\textsuperscript{16}, indicating that HF\textsubscript{1} is different from YF\textsubscript{1} and TF\textsubscript{1}.

In contrast to reports from TF\textsubscript{1}, significant variation was observed in the measured angles between the binding and hydrolysis dwells. At intermediate ATP concentrations, typically either three broad peaks—likely due to unresolved closely separated dwells—or six peaks with separations above 40\,\mu m were observed. To resolve closely separated peaks, flow cells were used to change the ATP concentration from 5\,mM to 3\,mM, and bead trajectories were analysed to compare the position of the dwells at low and high ATP concentration. Fig. 4A–D shows one such molecule captured before, during and after the flow of 3\,mM ATP. Typically, dwell kinetics were assessed before and after the addition of 3\,mM ATP (4B and 4D), see SI for details of the other molecules observed. Fig. 4E shows a histogram of the measured angles between the binding (5\,mM) and hydrolysis (3\,mM) dwells, as analysed using k-means clustering to identify the dwell locations at low and saturating ATP (Methods). A least squares Gaussian fit to the results from 18 molecules (54 dwell separations) gave a peak at 33\,\mu m with a width of 19\,\mu m (Fig. 4E). The median and mean separations were 31\,\mu m and 34\,\mu m. These measurements indicate that the separation between the binding and hydrolysis dwells in wild type yeast F\textsubscript{1} is similar to the separation previously measured in TF\textsubscript{1}\textsuperscript{34} and EF\textsubscript{1}\textsuperscript{6}, albeit the variation is larger.

The number of frames that rotating molecules spent in each dwell state for saturating (\textapprox 1\,mM) and low (\textless 10\,\mu M) concentrations of ATP were counted. A Bayesian information criterion (BIC)\textsuperscript{35} was used across models with 1, 2 or 3 sequential irreversible Poisson steps, both with and without a delay for bead rotation, to judge the quality of fit to each individual trace, as in previous work\textsuperscript{6}. At saturating ATP, the data fit best using a short delay (0.13 \pm 0.06\,ms) and two ATP-independent steps (rate constants 2.5 \pm 1.2\,(ms\textsuperscript{-1}), 4.3 \pm 2.3\,(ms\textsuperscript{-1}). At low ATP (3–10\,\mu M), the data fit best using one ATP-dependent step (18 \pm 6\,\mu M\textsuperscript{-1}\,s\textsuperscript{-1}) and one ATP-independent step with a rate (1.8 \pm 1.2\,(ms\textsuperscript{-1}) consistent with being an unresolved combination of the two steps fit at saturating ATP. Dwell distributions of a composite of 15 molecules at low and saturating ATP, and the best kinetic fits that result, are shown in Fig. 5. These
results suggest that the rotation of wild type yeast F1 is governed by one ATP-dependent process located at the ATP binding dwell and two ATP-independent processes at the catalytic dwell. Taken together, these results suggest that the fundamental kinetics of yeast F1 are similar to those of F1 from *E. coli* and the thermophilic *Bacillus* PS3, but different to those of human F1. That is, there is one ATP sensitive process located at the ATP dwell angle, then two ATP insensitive processes which occur following a 30–40° rotation of the central stalk. In addition, we observe a short delay at high ATP which is consistent with the expected time required for rotation of the gold bead following a mechanochemical step in F1.

**Analysis of “liver isoform” and six mutant forms.** We used the methods developed here to screen seven forms of yeast F1 containing a single point mutation relative to the wild type enzyme. One mutant ATP, and measuring the angle change between the vectors connecting dwell locations (blue triangle on x-y plots). (E) Histogram of 54 dwell angle separations observed from 18 molecules and least squares Gaussian fit to the histogram. The molecule shown in (A–D) produced atypically large angular deviations (48, 49, 64°), compared to population median of 31°.

Figure 4 | Dwell distributions for a composite of 15 wild type F1 molecules at (A) low (10 μM) ATP and (B) saturating (3 mM) ATP. The low ATP data are fit by two sequential Poisson processes, saturating ATP by two sequential Poisson processes with a time offset. The values for the kinetic rates and time offset from the fits of the composite dwell distributions agree with the kinetic rates and time offset calculated from the median values of single molecule fits within the 95 percent confidence intervals.
form corresponds to the “liver isofrom”, while six forms contain mitochondrial genome integrity (mgi) mutations.

Fig. 6 shows speed-ATP curves for these mutant forms, and for comparison the wild type control from Fig. 3. The values associated with Michaelis Menten fits to each mutant are summarised in Table 1. Data obtained from the “liver isofrom” mutant enzyme, γG278D, are indistinguishable from those of the wild type enzyme. This indicates that despite the tight regulation between the liver and heart isoforms, the Asp residue at the terminal end of the γ subunit has no measurable effect on kinetics of rotation under ATP hydrolysis. In contrast, all the data from the enzyme forms with mgi mutations show a significant reduction in the maximum rotation rate, by a factor of 2–8. However, the rotation rates of these mutant enzymes are only slightly affected at low ATP concentrations. There was a significant (at 95% confidence) decrease in the observed rate of ATP binding for the mutants βV279F, αN67I and αF405S, which had a binding rate approximately two thirds that of the wild type enzyme. Data of the mutant enzymes generally fit well to Michaelis Menten kinetics, with the exception of βR408I.

Kinetic analyses were performed on the dwell states for the six mgi mutant forms of the enzyme, as for the wild type, in order to determine the kinetics at low and saturating ATP. In all mgi mutant forms, the ATP binding dwell was best fit by a single exponential with an ATP-dependent rate constant assigned as kATP. The catalytic dwell was best fit with two ATP-independent steps and an offset, similar to the wild type. The results are summarised in Table 1. Consistent with the Michaelis Menten fits (Fig. 6), the kinetics of the ATP binding dwell were changed relatively little, and those of the catalytic dwell much more, for all mgi mutants with respect to wild type. The rate of one of the processes occurring at the catalytic dwell dropped by as much as 10-fold, while the rate of the other process remained relatively unchanged. By comparison to TF1, we expect that these rates correspond to hydrolysis of ATP and release of phosphate, but this kinetic analysis does not determine which rate is altered, nor whether the same rate is altered in all mutants.

Data from the βR408I mutant showed an additional dwell at high ATP. At 3 mM, the rate of ATP binding is approximately 60 (ms)^−1, which is too fast to be resolved in this study, and the expectation was this dwell would not be observed. Surprisingly, six dwells were clearly visible in the majority of high quality traces at both intermediate and saturating levels of ATP, as shown in Fig. 7A, despite the fact that kATP was not measurably different from wild type. Although these dwells were not typically observed at saturating ATP in the other forms of F1 investigated, a similar weak signal was occasionally present in traces from the wild type enzyme, as well as βR408G, αF405S and γI270T (Fig. 7B–E). However, due to the short duration in forms other than βR408I, and variability in its observation, we were unable to isolate and characterise this state. It is clear, however, that some process at the ATP binding angle was significantly slowed in βR408I even at saturating ATP concentrations. Accordingly, it is possible that a short, poorly resolvable ATP-insensitive dwell at the ATP binding angle may be a general feature of yeast mitochondrial F1 but amplified in the βR408I mutant form.

Discussion

This study provides the first single molecule rotation analysis of F1 from a species where multiple X-ray crystal structures are also available, and the first comparison between high-resolution single molecule results from different mitochondrial F1s. In S. cerevisiae F1 as in bacterial F1, three ATP-independent processes occur approximately 33° before ATP binding. Given the similarity of our results to those with TF1 and EF1, we ascribe these to bead transit, ATP hydrolysis and phosphate release. This is identical to TF1, except that the equivalent work on TF1 lacked the time resolution necessary to observe bead transit33,34. By contrast, ATP hydrolysis and phosphate release in mitochondrial HF1 occur 30° and 55° before ATP binding, respectively36. It is possible that small differences between the angles of hydrolysis and phosphate release in YF1, TF1 and EF1 will be resolved by future improvements in temporal and spatial resolution36, but at present it appears that HF1 is different from the others in this respect. It remains to be seen whether these differences between different forms of F1 will show any discernible pattern; our results indicate that neither bacterial/mitochondrial nor thermophilic/mesophilic sources uniquely determine the pattern of catalytic dwell angles.

This study also demonstrates that the power spectral bias of gold bead position is a powerful method for quick, systematic detection of rotating molecules, facilitating the screening of several mutant forms. The power spectra can also be used to obtain speeds from noisy data traces, giving results comparable to those obtained using methods which require higher signal-to-noise.
The heart and liver isoforms of the γ subunit differ only by the addition of a single Asp residue at the C-terminus in the liver form, predicted to form a salt bridge with a E that would slow the catalytic cycle. However, the results of the rotational studies here suggest this is not the mechanism of regulation, as the kinetics are unchanged. Instead, if the Asp serves in a regulatory mechanism, it likely occurs in conjunction with another molecule. The lack of observable effect of the C-terminal Asp is consistent with observations that crosslinking

| Form                  | MICHAELIS MENTEN FITS | Dwell analysis |
|-----------------------|-----------------------|----------------|
|                       | vmax (rev s⁻¹) | Km (μM) | kATP (s⁻¹ μM⁻¹) | n | kₐ₅P (s⁻¹ μM⁻¹) | k2 (ms⁻¹) | k3 (ms⁻¹) | n |
| Wild type             | 586 ± 24           | 62 ± 9 | 28.3 ± 3.6     | 2177 | 18 ± 6 | 2.5 ± 1.2 | 4.3 ± 2.3 | 18, 28 |
| γG278D                | 582 ± 137          | 63 ± 33 | 27.6 ± 10.2   | 89 | 26 ± 2 | 2.6 ± 0.6 | 5.0 ± 1.7 | 4, 5 |
| βV279F                | 144 ± 13           | 24 ± 5 | 17.9 ± 2.8     | 537 | 15 ± 2 | 0.36 ± 0.08 | 2.3 ± 2.7 | 4, 8 |
| αN671                 | 248 ± 47           | 41 ± 12 | 18.0 ± 2.6     | 432 | 14 ± 3 | 0.46 ± 0.09 | 2.3 ± 0.7 | 7, 3 |
| βR408G                | 80 ± 11            | 10.0 ± 2.6 | 24.1 ± 4.0   | 439 | 19 ± 5 | 0.21 ± 0.08 | 1.3 ± 0.5 | 10, 17 |
| αF405S                | 336 ± 40           | 49 ± 11 | 20.4 ± 3.2     | 1044 | 16 ± 6 | 0.8 ± 0.6 | 2.0 ± 0.9 | 62, 40 |
| γI270T                | 377 ± 92           | 44 ± 15 | 25.7 ± 4.4     | 2190 | 22 ± 7 | 0.8 ± 0.5 | 2.5 ± 2.0 | 109, 27 |
| βR408I                | 69 ± 13            | 7.9 ± 2.5 | 26.4 ± 4.7   | 6519 | 23 ± 6 | 0.20 ± 0.07 | 3.3 ± 2.3 | 384, 51 |
| Bacillus PS3³³        | 129 ± 22           | 15 ± 5 | 25.8           | 42 | 441 ± 39 | 21.1 ± 1.0 | 43 ± 4 |
| E. coli              | 129 ± 22           | 441 ± 39 | 21.1 ± 1.0   | 43 ± 4 |

The results of the analysis from the Michaelis-Menten fits and the dwell time histogram analysis for wild type and the seven mutants screened. All values are quoted with 95% confidence intervals, with the exception of Bacillus PS3 and E. coli, where the values were sourced from previous studies as indicated in the table. The errors in these studies were typically quoted as standard deviations; for comparison to the 95% CIs listed here they have been multiplied by 1.96 for data where the number of degrees of freedom were unknown, or by the appropriate t-statistic where the number of degrees of freedom was known (2.45 to 1.96 for df = 6 to 10). In some cases the error could not be calculated from the available data and has been left blank. The number of traces used is denoted by n. For the dwell analysis, the first value of n corresponds to the number of traces used to calculate kₐ₅P and the second value for the number of traces used to calculate k₂ and k₃.

Figure 7 | Six dwells at saturating ATP. (A) Six dwells are visible at saturating ATP for mutant βR408I. (B–E) Examples showing occasional dwells between major steps for (B) βR408G, (C) αF405S, (D) wild type and (E) γI270T. Note that while (A) is typical of data for βR408I, (substeps were detected by eye in only 1/21, 6/160, 3/45 and 5/360 high quality data traces for (B) βR408G, (C) αF405S, (D) wild type and (E) γI270T respectively. All traces shown were recorded at 10 kHz and 3 mM ATP, except (C) which was at 1 mM ATP. The expected ATP-dependent dwell is −1/6 frame at 3 mM ATP, and would not be resolved. Scale bar marks 40 nm.
the equivalent residue to the α-subunit changes neither the catalytic activity nor the torque generated by ATP hydrolysis37,38.

Six mtg mutant forms of S. cerevisiae F1-ATPase were studied to investigate molecular defects which uncouple the yeast ATP synthase29. Mutations that largely or completely uncouple the enzyme are technically very difficult to work with; all of the mtg mutations studied here are “leaky”, meaning that significant coupling is still present. The most consistent and significant change across all mutants was a substantial slowing of one of the processes, presumably ATP hydrolysis or phosphate release, at the catalytic dwell39. Another clear feature of our results is that the ATP binding rates observed at limiting [ATP] were 70–100% of the wild type rate, while the catalytic rates at saturating [ATP] were 15–50% of the wild type rate. This indicates that these mutations strongly affect either the rate of ATP hydrolysis or the rate of phosphate release, but have negligible effect on ATP binding.

mtg mutations have been categorized into two classes based upon their effect and location in the high-resolution structure of yeast F1 ATPase28. Class 1 mutations are located at the top of F1 (Fig. 1a) and form interactions with the E site at the Catch 1 region. The three Class 1 mutants studied here, γ1270T, αN671, and βV279F, showed no, minor, and significant conformational changes in the high-resolution crystal structure, respectively. Consistent with this pattern, the decrease in rotation speed was greatest for βV279F and smallest for γ1270T. The changes to the high-resolution crystal structures were primarily around the E site, and the structure of the mutant forms αN671 and βV279F indicated that, unlike the wild type enzyme40, phosphate was not bound in the E site, suggesting that the affinity for phosphate has been reduced. Given the minimal disruption to the DP site in the crystal structure, it is probable that the slow rate observed for Class 1 mutants corresponds to phosphate release. The combination of structural and kinetic data therefore suggests that both the rate of phosphate release and the affinity for phosphate are reduced in the mutant. This implies that the binding rate of phosphate may be greatly reduced, consistent with impaired ATP synthesis.

The Class 2 mutations (αF405S, βR408I and βR408G) also show a significant reduction in the rate of one of the processes occurring at the catalytic dwell. Given the structural similarity of the E site to wild type in structural data, it seems likely that ATP hydrolysis at the DP site, rather than phosphate release, is slowed in Class 2 mutants. αF405S, βR408I and βR408G are located at the bottom of F1, and while they are near the α/β interface, they do not change the conformation of the DP, TP or E active sites, but do alter the structure near the Catch 2 region. mtg residues αF405 and βR408 share a cation/π interaction33 and the crystal structure of αF405S shows a disruption in this interaction33. Both βR408G and βR408I also likely disrupt this interaction. The kinetic data for βR408I (and occasionally for βR408G and αF405S) showed an unexpected pattern of slow dwells at the ATP binding angle at saturating ATP. This slower dwell indicates a structural change in the conformation of F1 at the ATP binding dwell (in TP*, DP* or E*) and indicates a role for the cation/π interaction in one of the three reactions associated with the ATP binding dwell in bacterial F1; ATP binding, ADP release, or an unidentified temperature-sensitive step33,44. To discriminate between these three we note that the principal change in the crystal structure of αF405S is a disruption of the interactions between the γ and β subunits. Release of ADP occurs in the DP* state at or just after 240°, with rotation of the γ subunit greatly increasing the off-rate for ADP45. While ADP is being released from the DP* site, ATP is binding at the TP* site, and the two processes are co-ordinated by the γ subunit. We hypothesize that Class 2 mutations, as exemplified by αF405S, disrupt the αF405/βR408 cation/π interaction and interactions between the β and γ subunits that co-ordinate ATP binding and ADP release, and that the slowing of the binding dwell is due to the resulting decrease in the rate of ADP release.

Current crystal structures of F1 correspond to the catalytic dwell, and hence provide no direct structural information into the impact of Class 2 mutations on ADP release and the binding dwell. The speed of βR408I reduces with increasing [ATP] past Km, suggesting that conformational changes in the disrupted DP* site may be slowed and that ATP may weakly interact with the disrupted site. Alternatively, it is plausible that disruptions to coordination via the gamma subunit could delay the hydrolysis of ATP and allow rotation of F1 after phosphate release, but before ATP hydrolysis. The release of ADP would then be delayed until hydrolysis was complete. These predictions may be testable by computationally regenerating the ATP binding intermediate for Class 2 mutants.

Our single molecule data show no direct evidence of F1 moving into an uncoupled kinetic cycle where more than one ATP is consumed in a 120° rotation. However, the biochemical assays that show uncoupling involve situations where F1 experiences high load or is operated in the synthesis direction, and it is likely that these mutants remain tightly coupled when no load is present. Two technologies demonstrated on thermophilic F1 might be useful for investigating coupling – fluorescent ATP has been used to track ATP binding and ADP release from F143, and magnetic handles have been used with magnetic tweezers to rotate F1 in the synthesis direction44, with one suggestion that uncoupling (“slips”) are observable in the wild type thermophilic enzyme43. Furthermore, we have not measured torque output from these mutants, which might correlate with coupling. We expect further work along these lines will help elucidate the mechanism of uncoupling in these mutants. Further, a crystallographic goal is to obtain the structure of F1 in a number of additional intermediate conformations including the ATP binding dwell conformation. The results in this study illustrate the power of single molecule analysis, the limitations of x-ray crystallographic data, and the complementarity of the approaches.

**Methods**

Covalent Modification of F1. Yeast F1 was genetically engineered using the QuikChange method (Agilent Tech.) to include two surface cysteine residues, γD101C and γE189C and a His-tag on the amino end of the β-subunit, and was purified as described46. The cysteine residues were biotinylated by incubating: 10 mM (3.5 mg/ml) F1, 20 mM Biotin-PEAC5-maleimide (6-[N-[(2-[N-(Maleimidomethyl)-ethyl]-N-piperazinyldimethyl)ethyl]-Biotin hydrazide, hydrochloride, Dojindo Laboratories), 50 mM HEPES, pH 8.0, 2 mM ATP, 2 mM MgSO4 for 30 min at 23°C, quenching with 100 mM DTT, and desalting on a centrifug e column containing Biogel P6 resin (BioRad). The protein concentration was adjusted to 0.4 mg/ml in buffer containing 50% glycerol, flash frozen and stored in liquid N2. This modification does not alter the activity of the enzyme.

Gold bead preparation. 60 nm gold beads (BBInternational) were functionalised with either Neutravidin or Streptavidin. Neutravidin beads were prepared by combining gold beads with 8 mg/ml Neutravidin in 10 mM Tris pH 8.0, and storing at 4°C for a minimum of 12 hours49. Streptavidin beads were prepared on Ref. 48 by combining 5 μl each of 0.5 mM DSP, 10 mM TCEP and 100 μM streptavidin, each in 10 mM MOPS pH 7.0 with 50 mM KCl. This was incubated for five minutes, then 1 ml of gold beads was added as supplied, incubated for 2 hours at room temperature, and stored at 4°C. Beads were washed before use with excess 10 mM HEPES pH 7.0 (Neutravidin) or Tris pH 8.0 (streptavidin), followed by 10 mM MOPS pH 7.65, 50 mM KCl, 2 mM MgCl2.

Rotation assays. Glass coverslips were functionalised with Ni**+**-NTA as described previously46. Tunnel slides used double-sided tape to define a channel between a microscope slide and a Ni**+**-NTA functionalised coverslip. Where the buffer was changed during microscopy, flow slides were used as described in SI, with two short tubes and the input tube connected to a syringe. F1 (1–10 nM in buffer A - 10 mM MOPS, 50 mM KCl, 2 mM MgCl2, 10 μM ATP, pH 7.65) and then gold beads (in buffer B = buffer A + 1 mg/ml BSA for streptavidin beads, or buffer A + 10 mg/ml Neutravidin beads) were added for ten minutes and washed with at least 5 volumes of buffer B. Rotation was observed in assay buffer (buffer B + ATP 1 μM – 3 mM, 2 mM phosphate and 20 units/ml pyruvate kinase). Where the concentration of ATP in the assay buffer was less than 10 μM, the ATP concentration of buffers A and B was reduced to the same level.

Tunnel slides were viewed under a high-speed backscattering darkfield microscope34 and images of multiple gold beads recorded at frame rates between 60 Hz and 30 kHz using a CMOS camera (Fastcam 1024 PCI, Photron) with 85 nm per pixel. For flow slides a custom stage with high mechanical stiffness replaced the piezo mount described in Ref. 50.
Temperature. Microscope temperature was measured using a K-type thermocouple, and was 23.0 ± 0.5 °C for all experiments used for speed or kinetic rate measurements. For flow slide experiments, used to measure the angle separation of substeps, the temperature was 22.5 ± 1.0 °C.

Video Analysis. Analysis of bead motion was performed using custom-written MATLAB software, as follows (see SI for details). Bead positions and intensities in each frame were calculated using a Gaussian mask algorithm. Spinning beads were identified from directional bias in bead rotation, where \( PSD(o) \) is the power spectral density at angular frequency \( o \) of the bead’s position \( (x(t), y(t)) \) expressed as the complex number \( z = x + iy \), the sampling rate and \( L \) is a low frequency cutoff used to remove noise associated with microscope drift, typically set to the lower of 5% of the total rotation speed under the assay conditions used or 10 Hz. Beads identified as duplicates based on either radius of motion or intensity, were removed from the data set for all speed measurements and for kinetics measurements at high ATP (where the extra drag is significant). The software allows screening a sample to reliably detect rotating probes prior to acquisition of long videos.

Rotation speed. Ellipses were fit to the \( x-y \) data using an algebraic algorithm, from which an unwarped angular position was derived. Pauses, during which a bead stopped rotating, were identified as described below in the sub-section "kinetic fits". Angular speed was estimated as the total angular displacement during the video divided by the total time, having first removed any pauses. Beads were excluded from speed measurements if more than 2% of the data points were closer to the centre of the fitted ellipse than one-third of the radius of the ellipse. Traces rejected by this criterion tend to have multiple false centre-crossings, and produce measured speeds that are lower than the actual rotation speed, as shown in Fig. 2.

Substep angle measurements. Data were collected using a flow slide, observing the same field of molecules at low followed by saturating ATP concentrations. Comparison of videos collected before, during, and after buffer exchange, showed that there was typically translation of the field of view associated with flowing buffer through the slide, but no detectable rotation of the field of view.

For each molecule rotating before and after the buffer exchange, the last spinning episode before, and the first spinning episode after buffer exchange were selected. Episodes were discarded where low ATP speed was greater than 50 Hz or the final speed was less than 400 Hz (to ensure full buffer exchange), or the ratio of the long to short axis of the fit ellipse was greater than 1.5 (when angle recovery is less accurate). Measuring angles via ellipse fits before and after buffer exchange was unreliable, as a misplacement of the ellipse fit by a few nm significantly distorted the recovered angles. Instead, k-means clustering with 3 clusters was used to identify the dwell locations \( (x, y) \) at low and high ATP. The vectors between successive dwell states were analyzed for kinetics measurements at high ATP (where the extra drag is significant). The software allows screening a sample to reliably detect rotating probes prior to acquisition of long videos.

Kinetic fits. Kinetics were fit to angle-time traces in a manner similar to previously. For fitting three dwell states per revolution, the angle trace was unwrapped and broken into 120° segments, and the number of data points in each segment was used to generate a histogram of dwell times, pooling dwells from all 3 states. Pauses were removed by ignoring dwells longer than 7 times the mean dwell length. For single exponential kinetics this corresponds to removing the longest 0.1% of real (i.e., non-pause) dwells and a 0.6% (i.e. negligible) systematic underfit of the rate constants; for other kinetic model the fraction of real events removed and effect on the fit is smaller. The dwells immediately preceding and following pauses were also removed.

A histogram of the dwell times was constructed for each enzyme. Five kinetic models were considered as potential fits to the histogram, comprised of 1, 2 or 3 kinetic rates per dwell both with and without a rotation-delay time offset. The dwell time histograms were fit to each model and rate constants were derived with a maximum likelihood estimation (MLE) algorithm. The likelihood of these six models and thus the quality of fit was compared using the Bayesian information criterion (BIC).
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**Author contributions**

B.C.S. collected all experimental data. Y.W. and V.P. performed all genetic transformations, enzyme purifications, and biotinylation modifications. B.C.S. and A.L.N. were responsible for software, algorithms and analysis. A.L.N., B.C.S., D.M.M. and R.M.B. wrote the manuscript.

**Supplementary information**

accompanies this paper at http://www.nature.com/scientificreports

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