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The nucleotide binding affinities of two critical conformations of *Escherichia coli* ATP synthase

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Abstract

ATP synthase is essential in aerobic energy metabolism, and the rotary catalytic mechanism is one of the core concepts to understand the energetic functions of ATP synthase. Disulfide bonds formed by oxidizing a pair of cysteine mutations halted the rotation of the γ subunit in two critical conformations, the ATP-waiting dwell (α E284C/ γ Q274C) and the catalytic dwell (α E284C/ γ L276C). Tryptophan fluorescence was used to measure the nucleotide binding affinities for MgATP, MgADP and MgADP-AlF₄ (a transition state analog) to wild-type and mutant F₁ under reducing and oxidizing conditions. In the reduced state, α E284C/ γ L276C F₁ showed a wild-type-like nucleotide binding pattern; after oxidation to lock the enzyme in the catalytic dwell state, the nucleotide binding parameters remained unchanged. In contrast, α E284C/ γ Q274C F₁ showed significant differences in the affinities of the oxidized versus the reduced state. Locking the enzyme in the ATP-waiting dwell reduced nucleotide binding affinities of all three catalytic sites. Most importantly, the affinity of the low affinity site was reduced to such an extent that it could no longer be detected in the binding assay ($K_d > 5$ mM). The results of the present study allow to present a model for the catalytic mechanism of ATP synthase under consideration of the nucleotide affinity changes during a 360° cycle of the rotor.

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Conflict of interest

The authors declare that there is no conflict of interest.

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Keywords

ATP synthase; Disulfide crosslink; Nucleotide binding; Catalytic mechanism; Tryptophan fluorescence; Rotational catalysis

1. Introduction

ATP synthase is structurally and functionally conserved among species. ATP synthesis through oxidative phosphorylation or photophosphorylation plays a critical role in the energy metabolism of animals, plants and many microbes [1]. The prototype enzyme, ATP synthase from *Escherichia coli* consists of eight different subunits: three are found in the membrane-embedded F_0 subcomplex (ab_2c_{10}), and five are found in the water-soluble F_1 subcomplex ($\alpha_3\beta_3\gamma\delta\epsilon$) [2]. ATP synthase couples ATP synthesis/hydrolysis and the movement of protons across the membrane. The conversion of electrochemical energy (proton gradient) to chemical energy (ATP) occurs in ATP synthase through its unique rotary catalytic mechanism, with mechanical energy as intermediate [3]. Protons flowing down the electrochemical gradient push the rotor complex ($c_{10}\gamma\epsilon$) to spin. Rotation of the γ subunit within the catalytic hexamer ($\alpha_3\beta_3$) changes the conformation of three catalytic binding sites on the β subunits to synthesize ATP from ADP and inorganic phosphate (P_i) [4]. In bacteria, under certain physiological conditions the enzyme can run in reverse, hydrolyzing ATP to build up a proton gradient which the bacterium needs for nutrient uptake and locomotion [5]. For further reviews see Refs. [6–10].

According to Boyer's "binding change" principles, in ATP synthesis, the energy obtained from the proton gradient is used to reduce the affinity of MgATP as well as to increase the affinity for substrates, but not for the chemical synthesis step itself [11]. The rotational angle of the γ subunit determines the conformation of each of the three β subunits, which have been named β_{TP} (AMP-PNP bound), β_{DP} (ADP bound) and β_E (empty) according to the nucleotide occupancy in the original crystal structure, with the nucleotide-bound sites in the "closed" conformation and the empty site in the "open" conformation [12]. The catalytic binding sites located in these β subunits have vastly different affinities to Mg^{2+} -nucleotides (thus named high, medium or low affinity site) [13]; a "half-closed" conformation (β_{HC}) is proposed to be the intermediate state between open and closed conformation [14]. The position of γ determines which site has high, medium, or low affinity at any given point of time. Given that three MgATP molecules are synthesized or hydrolyzed during a full revolution of the γ subunit, each 120° rotation step is identical [15]. Viewing from the ATP hydrolysis direction, a 120° step can be further resolved into an 80° substep for MgATP binding and a 40° substep for release of products MgADP and P_i [6,16–18]. The pause before the 80° substep is named "ATP-waiting dwell", and the time of this dwell is dependent on the MgATP concentration (< 0.1 ms under MgATP saturation). A lag time has been observed before the 40° substep as well; during this time, MgATP is hydrolyzed. This "catalytic dwell" lasts approximately 2 ms and it is independent of the MgATP concentration [19].

The energy necessary to drive rotation in hydrolysis direction is not provided by MgATP hydrolysis itself, but by changes in the binding affinities of substrate and products [1,4,20,21]. Mapping the profile of nucleotide binding affinities as a function of the rotational angle of the γ subunit is essential for complete understanding of the rotary catalytic mechanism. In this study, we use tryptophan fluorescence to measure nucleotide binding affinities after locking the enzyme by disulfide crosslinking [22] in the two most important conformations: in the ATP-waiting dwell and in the catalytic dwell. The outcome will give further insights into the molecular basis of the catalytic mechanism of ATP synthase.

2. Materials and Methods

2.1. Strains

E. coli strain DH5 α (New England BioLabs) was used as the competent cell for site-directed mutagenesis. Bearing no intrinsic ATP synthase operon, *E. coli* strain DK8 ($\Delta(atpB-atpC)$, tetracycline resistant) [23] was used to express ATP synthase from the respective plasmid.

2.2. Construction of plasmids

Plasmid pSN6 (pBR322 derivative, *atpB-atpC*, β Y331W, ampicillin resistant) [24] was used as the template throughout this study and is referred to as WT (“wild-type”) in the following text. Mutations were introduced by site-directed mutagenesis strategy (oligonucleotides from Integrated DNA Technology, listed in Supplementary Material Table S1; Q5® High Fidelity DNA Polymerase from New England BioLabs). A plasmid with desired mutation was verified by restriction pattern analysis and DNA sequencing, followed by transformation into strain DK8 for further assays.

2.3. Growth yield assay

A single colony of DK8 strain expressing WT or mutant ATP synthase was inoculated in lysogeny broth (LB) medium with 100 μ g/ml ampicillin and grown to late exponential phase. A further 1:500 inoculation was made into medium containing 8 mM succinate or 4 mM glucose [25]. Cells grew aerobically at 30 °C until saturation. Growth yield was quantified from turbidity of the liquid culture by measuring its absorbance at 590 nm. Each mutant was assessed at least in triplicate.

2.4. Inverted membrane vesicle preparations

Cells grew aerobically at 30 °C and then were harvested at late exponential phase. Buffers used in experiments were prepared as previously described [26]. Resuspended cells were lysed mechanically by two passes through a homogenizer (EmulsiFlex-C3; Avestin, Ottawa, Ontario, Canada) at 15 kpsi [27]; after removal of cell debris by ultracentrifugation, membrane vesicles were pelleted from the supernatant and purified. For each strain expressing WT or mutant ATP synthase, at least two biological replicates were prepared.

2.5. F₁ subcomplex protein purification

The water-soluble F₁ subcomplex of WT and mutants was purified as previously described [28]. In brief, membrane vesicles were washed three times in the buffer of low ionic strength, causing the F₁ subcomplex to dissociate from the membrane. F₁ was precipitated using polyethylene glycol 6000 (PEG, 10% final concentration). Resuspended F₁ was purified by anion-exchange chromatography on Whatman DE52 cellulose with a stepwise elution with Na₂SO₄. F₁ was concentrated by ultrafiltration and further purified by size exclusion chromatography on Sephacryl S-300 HR. Fractions with high specific ATPase activity were pooled and concentrated. F₁ was stored at – 80 °C.

2.6. Protein concentration assay

The Bradford method was used to determine protein concentrations, with bovine serum albumin as standard [29]. All samples were measured at least in duplicate.

2.7. Western blot

The relative expression amount of membrane-bound ATP synthase was quantified through Western blot [30] with anti- γ antibody (a kind gift from Drs. Toshiharu Suzuki and Masasuke Yoshida, Japan Science and Technology Agency, Tokyo). Band intensity was evaluated by ImageJ software (National Institutes of Health). Two biological replicates were assessed.

2.8. Disulfide bond crosslinking and reduction

The disulfide bond was formed between two cysteine residues by 1 mM CuSO₄ oxidation for one hour at 25 °C; to reduce a disulfide bond, protein samples were treated with 1 mM (or 4 mM, in the recovery assay) dithiothreitol (DTT) for one hour at 25 °C [31]. The concentration of membrane protein as well as the F₁ samples was adjusted to 1 mg/ml to ensure the efficient oxidation or reduction.

2.9. ATPase activity assay

ATPase activities were assessed at 30 °C in a medium containing 50 mM Tris/H₂SO₄, 4 mM MgSO₄ and 10 mM ATP, pH 8.0. To trigger ATP hydrolysis, inverted membranes (10 µg/ml) or purified F₁ proteins (5 µg/ml) were added into the medium. To terminate the reaction, sodium dodecyl sulfate (SDS) was added to a final concentration of 5% (w/v). Inorganic phosphate released from the reaction was quantified colorimetrically by measuring the absorbance at 700 nm [32]. Each protein sample was assayed at least in duplicate.

2.10. Proton pumping assay

Inverted membrane vesicles (100 µg) were vigorously suspended in 2.0 ml proton pumping medium containing 10 mM HEPES, 300 mM KCl, 5 mM MgCl₂, 1 µg/ml valinomycin and 1 µM acridine orange, pH 7.5, at 25 °C. The acridine orange fluorescence intensity was measured at an emission wavelength of 530 nm by using an excitation wavelength of 460 nm. To initiate proton pumping, 1 mM ATP was added; 5 µM carbonyl cyanide *m*-chlorophenylhydrazone (CCCP) was added to dissipate the proton gradient [33]. Each sample was assayed at least in duplicate.

2.11. Nucleotide binding assay

As F_1 is purified and stored in buffer containing MgATP, it may have nucleotides bound to the catalytic sites [34]. To remove them, F_1 samples were subjected to chromatography with Sephadex G-50 directly before the nucleotide binding assay [35]. Nucleotide binding to the catalytic sites was measured using the fluorescence of $\beta Y331W$. This technique is described in detail in [13,28], addressing issues such as the precautions taken to keep ATP hydrolysis in MgATP binding experiments to a minimum (see also Supplementary Material) and justification of the assumption that all three catalytic sites contribute equally to the total fluorescence signal. Using a spectrofluorometer type FL3-22 (Jobin-Yvon/Horiba), the excitation wavelength was set to 295 nm, and the intensity at 350 nm from emission spectra was recorded [13]. The assay solution consisted of 50 mM Tris/H₂SO₄ (pH 8.0), 40 nM of F_1 and 2.5 mM MgSO₄. ATP or ADP was added from 30 nM to 5 mM. For titration with the transition state analog MgADP-AlF₄, the assay solution contained in addition 1 mM AlCl₃ and 10 mM NaF [36,37]. It should be noted that binding of MgADP-AlF₄ is reversible, with a half-life of inhibition of approximately 100 h [37]. WT F_1 ($\beta Y331W$) was used as positive control. True wild-type F_1 ($\beta Y331$) was used as reference to correct for the inner filter effects at high concentration of nucleotides [28]. At least three assays were done for each data point. To calculate the nucleotide binding affinities, data collected were fit to the following four equations.

$$Q = 1 - \left[\left(\frac{F_{exp}}{F_0} \right)_{Sample} / \left(\frac{F_{exp}}{F_0} \right)_{Reference} \right] \quad (\text{Eq. 1})$$

$$n = \frac{3Q}{Q_{sat}} \quad (\text{Eq. 2})$$

$$[L]_{Free} = [L]_0 - n \times [E]_0 \quad (\text{Eq. 3})$$

$$n = \frac{[L]_{Free}}{[L]_{Free} + K_{d1}} + \frac{[L]_{Free}}{[L]_{Free} + K_{d2}} + \frac{[L]_{Free}}{[L]_{Free} + K_{d3}} \quad (\text{Eq. 4})$$

In these equations: Q is the quenching of fluorescence intensity, and Q_{sat} is the quenching of fluorescence intensity when all three catalytic binding sites are occupied. As an exception, as titrations of the $\alpha E284C/\gamma Q274C$ mutant in the oxidized state did not reach the same degree of quenching as the reduced state, Q_{sat} of the reduced state was also used for the oxidized state. F_0 is the initial fluorescence intensity of proteins, and F_{exp} is the fluorescence intensity upon nucleotide titration. $[L]_0$ is the total nucleotide concentration (with an excess of Mg²⁺, all ATP or ADP was assumed to be in form of a Mg-nucleotide complex), $[L]_{Free}$ is the free nucleotide concentration (the concentration of Mg-nucleotide not bound to the enzyme), which can be calculated based on the total ligand concentration $[L]_0$, the total concentration of F_1 , $[E]_0$ (40 nM in this study), and the number of occupied catalytic binding sites, n (Eq. 3). K_{d1} , K_{d2} and K_{d3} are the dissociation constants of the three catalytic sites.

2.12. Miscellaneous

SigmaPlot (Systat Software Inc.) was used for non-linear least-squares regression.

3. Results

3.1. Functional characterization of cysteine mutants

A previous study [22] had shown that an α E284C/ γ L276C disulfide bond halts rotation in the catalytic dwell, while an α E284C/ γ Q274C bond freezes the enzyme in the ATP-waiting dwell. Thus, we introduced the single mutations α E284C, γ Q274C and γ L276C and the double mutations α E284C/ γ Q274C and α E284C/ γ L276C into *E. coli* ATP synthase. The mutant enzymes were characterized initially by growth yield in medium containing limiting glucose or succinate as sole carbon source to study the effect of the mutations on ATP synthesis; the effect on ATP hydrolysis was determined using ATPase activity and ATP-driven proton pumping of membrane preparations (Table 1). For the *in vitro* assays (ATPase, proton pumping; Table 1, columns 5 and 8), the membranes were pretreated with 1 mM DTT for one hour at room temperature to reduce any disulfide bond possibly formed by spontaneous oxidation.

Mutation γ Q274C or γ L276C did not significantly affect the functions of ATP synthase. Mutation α E284C resulted in moderate growth yield (62 – 72% of WT), impaired proton pumping ability (26 – 45% of WT) and low ATPase activity of membrane preparations (< 20% of WT). The behavior of the double mutants essentially reflected the effects observed with the single mutants (Table 1). The impairments of the α E284C mutant were not caused by reduced expression or reduced oligomeric stability (see the Western blot results in column 4 of Table 1). The amino acid residues at position γ 274 are not conserved, with charged (K and R), polar (T, N and Q) or small (G) residues (Supplementary Material Table S2); a cysteine in this position does not appear to cause a problem. Position γ 276 shows large aliphatic hydrophobic residues (I, L) in most species; replacement by a cysteine appears to be well tolerated. The glutamate residue at position α 284 is highly conserved, which might explain the impairment of enzyme function in the α E284C mutant. Unexpectedly, after isolation of the F_1 -ATPase subcomplex, the double mutants containing the α E284C mutation had WT-like ATPase activities (see 3.2. and Table 2).

3.2. The disulfide bond formed between subunits α and γ abolishes enzymatic activity by stopping rotation

F_1 -ATPase was purified from WT, α E284C/ γ Q274C, and α E284C/ γ L276C enzymes. The ATPase activity of these samples was assayed directly after preparation without any further treatment, after reduction of disulfide bonds with 1 mM DTT, or after oxidation of disulfide bonds with 1 mM CuSO_4 . With no treatment or after reduction by DTT, all three enzymes showed ATPase activities of 4 – 6 units/mg protein. Oxidized by 1 mM CuSO_4 , the ATPase activity of WT F_1 remained high (5.7 unit/mg protein); however, with the α - γ disulfide crosslink formed, ATPase activities of mutant F_1 with double cysteine substitutions almost dropped to zero (Table 2). With a disulfide crosslink formed upon oxidation, in the cysteine double mutants, α E284C/ γ Q274C and α E284C/ γ L276C, ATP hydrolysis was abolished since the γ subunit could no longer rotate; the residual ATPase activities might be due to a

marginal amount of enzyme with no α - γ disulfide bond or due ATP hydrolysis by the uni-site mechanism [38]. Furthermore, the data show that even in absence of treatment with DTT, the disulfide bonds between α and γ are in the reduced state.

The experimental data obtained with the WT enzyme confirmed that the treatment conditions for oxidation or reduction used in this study did not affect the hydrolytic capability. Still, the remote possibility remained that one of the introduced cysteine residues by itself might cause inhibition of the enzyme upon oxidation. We tested therefore the impact of oxidation on membrane ATP synthase samples with single cysteine mutations (Table 1, column 6), and they did not show any significant impairment compared to the reduced sample (Table 1, column 5). As observed for isolated F_1 , the double mutants were impaired due to formation of an α - γ disulfide crosslink. Full ATPase activity could be recovered by removal of the crosslink by re-reduction using an excess of DTT (Table 1, column 7). It should be noted that reductive removal of the crosslink also resulted in resuming rotation [22], a clear indication of enzymatic activity. These findings demonstrate that oxidation of the cysteines is reversible and does not cause any damage to the enzyme.

3.3. Nucleotide binding affinities of the WT and cysteine double mutant F_1

Following reduction or oxidation treatment, WT and mutant F_1 samples were titrated with MgATP, MgADP or MgADP-AlF₄ (a transition state analog). Quenching of fluorescence intensities of the tryptophan probe (β Y331W) reflects the occupancy of the catalytic binding site [13]. The results are summarized in Fig. 1 and Table 3. For WT F_1 , neither reduction nor oxidation treatment significantly affected the binding parameters of the catalytic sites. For comparison, previous studies [39,40] with untreated enzyme gave values for K_{d1} , K_{d2} , and K_{d3} of 0.02 μ M, 1.5 μ M, and 29 μ M for MgATP; 0.04 μ M, 6.0 μ M, and 42 μ M for MgADP; and < 1 nM, 0.06 μ M, and 40 μ M, for MgADP-AlF₄.

In the reduced state, the α E284C/ γ L276C double mutant F_1 exhibited a nucleotide binding pattern similar to WT. The affinities of site 2 for the transition state analog MgADP-AlF₄ and of site 3 for all tested nucleotides appeared to be slightly reduced by the mutations. More importantly, after oxidation to crosslink the rotor and the stator in the catalytic dwell state, the nucleotide binding parameters remained unchanged.

The α E284C/ γ Q274C double mutant had an overall reduced affinity for nucleotides. This seems to be mostly due to the γ Q274C mutation and not due to α E284C, as it was not observed for the α E284C/ γ L276C mutant to a similar extent. Still, in the reduced state α E284C/ γ Q274C F_1 showed the typical nucleotide binding pattern with three sites of different affinities. The α E284C/ γ Q274C mutant was the only enzyme investigated here that exhibited pronounced difference in nucleotide binding behavior between reduced and oxidized state. In the oxidized state, the affinities of the catalytic sites were so far reduced that at the highest MgATP concentration used (5 mM), the quench of β Y331W fluorescence was only about 2/3 of the value observed in the reduced state. Thus, locking of γ in the ATP-binding dwell results in the “loss” of a catalytic site, or, more precisely, K_{d3} becomes > 5 mM for all nucleotides tested. Furthermore, the affinities of sites 1 and 2 were reduced by factors of 5 – 29 in the oxidized versus the reduced state.

4. Discussion

4.1. Nucleotide binding pattern as a function of the rotational angle of γ

A complete description of the catalytic mechanism of ATP synthase will have to include an analysis of the affinity changes for substrates and products at each of the three catalytic binding sites during rotation; the affinities of the catalytic sites are determined by rotational angle of the central γ subunit. In the present study, we determined the nucleotide binding affinities of the catalytic sites in the two most important conformations in the catalytic cycle, the ATP-binding dwell and the catalytic dwell. For this purpose, we made use of two cysteine double mutants, α E284C/ γ L276C and α E284C/ γ Q274C, which upon oxidation to form a disulfide bond lock the enzyme in the catalytic dwell and the ATP-binding dwell, respectively [22], and we measured the nucleotide binding affinities using the fluorescence of a tryptophan introduced at position β 331 as signal [13,28]. The most important result of this study was the finding that locking the enzyme in the ATP-binding dwell gave an overall reduction in nucleotide binding affinities; the affinity of site 3 (the lowest-affinity site) was reduced so far that it could not be detected anymore with the technique used ($K_{d3} > 5$ mM).

It is highly unlikely that formation of the α E284C/ γ Q274C disulfide bond *per se* would lead to complete fluorescence quenching of β Y331W at the third catalytic site. The three tryptophan residues in position β 331 have a relatively high quantum yield (0.22) [28]. Complete quenching of the fluorescence requires a drastic change in their environment; formation of a π - π stacking complex between the tryptophan indole group and the adenine base of bound nucleotide provides such a change, which is the basis of the technique used here to study nucleotide binding to the catalytic sites [13,28]. The crystal structure of the *E. coli* enzyme [41] and those of the mitochondrial enzyme [12,14] show that, except for the absence or presence of nucleotide, all three β Y331 residues are in a very similar environment. Furthermore, except for the nucleotide, there is no other possible known quenching group nearby, such as the amide groups of glutamine and asparagine, the carboxyl groups of glutamic and aspartic acids, a lysine ϵ -amino group, a tyrosine phenol, a cysteine sulfhydryl, a histidine imidazole or a disulfide bond [42–44]. Thus, all fluorescence responses of β Y331W (or the lack thereof) have to be attributed to solely to the presence (or absence) of bound nucleotide, and not to any other independent quenching phenomenon.

4.2. Integration into mechanistic model

The new findings can be integrated into the previously-proposed mechanism for ATP hydrolysis by ATP synthase [15] as follows (Fig. 2). As in the previous version, the revised scheme takes into account (a) that in time-average, all three catalytic sites of *E. coli* ATP synthase have to be occupied by nucleotide to achieve significant catalysis rates [13,28,45]; (b) that under substrate saturation the MgATP to MgADP distribution is 1:2 [46]; (c) that the high-affinity site is the catalytically active site [47]; (d) that under substrate saturation virtually all enzyme molecules are in the catalytic dwell [19,48]; and (e) that the β_{TP} site is the high-affinity site [15]. While the last point is not universally accepted (for a different point of view, see e.g. [8]), the results of the present study strengthen the argument for the assignment of the β_{TP} site as the high-affinity site. Fluorescence resonance energy transfer experiments showed that residue γ 262 in the C-terminal helix of γ was much closer to the

high-affinity site than residue $\gamma 5$ in the N-terminal helix [15]. In the crystal structures [12,14], residue $\gamma 262$ is much closer to the β_{TP} site than $\gamma 5$. The present study confirms that the distance measurements were actually performed in an F_1 conformation similar to that seen in the crystal structures (see part 4.4.).

The revised rotary mechanism presented in Fig. 2A additionally takes into consideration the findings of the present study, (i) the observation that, unless the enzyme is locked in the ATP-binding dwell, the nucleotide binding affinities measured by the tryptophan fluorescence assay represent the enzyme in the catalytic dwell, and (ii) the newly-determined affinities for the ATP-binding dwell. Fig. 2B shows the changes in nucleotide binding affinity during the progress of catalysis. The K_d values for the ATP-binding dwell (the sites are labeled with an asterisk) are corrected for the affinity decrease due to the $\gamma Q274C$ mutation itself, even without oxidation.

After release of the reaction products from the previous catalytic cycle, the enzyme is in the ATP-waiting dwell (defined as 0°), with an empty low-affinity site (state A in Fig. 2B). The respective β subunit is in the open conformation with a K_d for MgATP of > 5 mM. At saturating MgATP concentrations, MgATP is immediately bound to this site (state B), which provides the energy to close the conformation of the β subunit, which in turn drives a rotation of γ by 80° , converting the low-affinity site into the high-affinity site (for further discussion, see Chapter 4.4. below). The enzyme is now in the 80° catalytic dwell and MgATP is bound very tightly ($K_d(\text{MgATP}) < 10$ nM by tryptophan fluorescence ([13,28] and this study); uni-site experiments gave a number of 0.2 nM [49]) (state C). MgATP is hydrolyzed at the end of the catalytic dwell [19] (state C $\rightarrow$ D); MgATP hydrolysis might actually trigger the subsequent 40° rotation step [50]. At 120° the enzyme is again in the ATP-waiting dwell (state E). Compared to the 80° catalytic dwell, the affinity is reduced by five- to seven-fold ($K_d(\text{ox})/K_d(\text{red})$ for $\alpha E284C/\gamma Q274C$), which would give K_d values between 1 and 50 nM for the WT enzyme. After the next 80° rotation step, at 200° , γ is back in the catalytic dwell with a K_d in the range of $1 - 10$ μM ([13,28] and this study). Continuing rotation reduces the affinity further (Fig. 2B), finally reaching a K_d of > 5 mM at 360° and allowing release of the reaction product MgADP. The techniques developed in our laboratory do not give us any insight into the timing of P_i release. There is considerable evidence that P_i is released during the 40° substep (state D $\rightarrow$ E) [51,52], as suggested in the scheme in Fig. 2A. However, the sequence of MgADP and P_i release is strictly hypothetical, as arguments for either scenario have been presented [51–53]. For an overview of residues involved in phosphate binding, see [54].

4.3. Comparison with literature data

The present study is not the first attempt to quantify the γ -angle dependence of the affinity of the catalytic sites. Two previous studies [55,56] measured on- and off-rates in single molecule experiments with the enzyme of *Geobacillus stearothermophilus* (formerly known as *Bacillus sp.* PS3) to calculate affinities; both agree with our results that the affinity of a nucleotide, newly-bound in the ATP-waiting dwell, will increase with the rotation angle of γ . Watanabe et al. [55] measured the affinity for MgATP at angles between -30° and $+30^\circ$ around the ATP-waiting dwell and found a continuous increase in affinity, with a K_d of

around 200 nM at -30° (equivalent to 330°) to 10 nM at 0° to 1 nM at 30° . The numbers raise the question of which binding site is monitored in these experiments; the highest ATP concentration used was 200 nM. MgATP hydrolysis at physiological rates requires that all three catalytic sites are participating. The K_m for MgATP hydrolysis in this enzyme is at least 7 μM [57]; at 200 nM ATP, less than 3% of all enzyme molecules will work in the physiological mode. Adachi et al. [56] used Cy3-ATP and Cy3-ADP as ligands in a rather ambitious project trying to cover all angles between 0° and 360° ; in the end, there were some gaps. Similar to our results, the affinity for Cy3-ATP increased from 0° to 80° (K_d 1 μM and 30 nM, respectively) and then dropped off. In contrast to our observation that the affinity is lowest at 0° (360°), the Cy3 nucleotides reach minimum affinity at 320° (K_d 30 – 40 μM). Another difference is the range of affinity changes. For Cy3 nucleotides it is 3 – 4 orders of magnitude; our study shows nearly twice as much. It is not quite clear if the Cy3-nucleotides are true representatives of the natural ligands/substrates; the fluorescent “Cy3” attachment is actually larger than the nucleotide. The authors state that the Cy3-attachment reduces on- and off-binding rates [56], but they do not give a K_m for bulk Cy3-ATP hydrolysis.

4.4. Open questions

The results of the present study leave some open questions. Most importantly, how can the open binding site with a K_d of > 5 mM efficiently bind the substrate MgATP when the K_m for MgATP hydrolysis is around 30 μM [13]. Here, thermal fluctuations appear to play a significant role [22,58,59]. Sielaff et al. [22] show that the fluctuations are actually larger in the ATP-waiting dwell and in the catalytic dwell during rotation than in the enzyme stalled by disulfide crosslinking. At some point in time, the γ subunit will be at a forward angle sufficiently large to increase the affinity of the open binding site so far that it can bind the substrate effectively (indicated by the red L** in Fig. 2A state B and in Fig. 2B).

Another open problem is the role of the ATP-waiting dwell. Sielaff et al. [22] demonstrated that nucleotide (MgADP) saturation gives an enzyme with γ in the position of the catalytic dwell. The results of the present study show that in absence of nucleotides on the catalytic sites γ must also be in the catalytic dwell position, as locking the enzyme in this position by disulfide crosslinking of the $\alpha\text{E284C}/\gamma\text{L276C}$ mutant had no effect on MgADP binding to the first (high affinity) site. In contrast, locking the enzyme in the ATP-waiting dwell with the $\alpha\text{E284C}/\gamma\text{Q274C}$ mutant resulted in a significantly reduced affinity. This finding agrees with the observation that in the crystal structure of the yeast enzyme in absence of nucleotide [60] the portion of γ within the $\alpha_3\beta_3$ cylinder is in essentially the same position as in the structures containing nucleotides [8,12]. The position of γ in the crystal structure bears more resemblance to the catalytic dwell than to the ATP-waiting dwell [22,61]. Thus, it seems that the ATP-waiting dwell just exists as an intermediate in the catalytic cycle. This agrees with the observation that the catalytic dwell position is thermodynamically favored [62].

4.5. Catalytic properties of the αE284C mutation

Finally, all mutants containing the αE284C exhibited a peculiar behavior that deserves a comment although it has little to do with the main topic of the present study. The ATPase

activity of purified F_1 from the $\alpha E284C/\gamma Q274C$ or $\alpha E284C/\gamma L276C$ double mutants in the reduced state was comparable to the WT enzyme (Table 2); however, the ATPase activity of membrane-bound $\alpha E284C/\gamma Q274C$ or $\alpha E284C/\gamma L276C$ ATP synthase in inverted membrane vesicles reached less than 20% of the WT activity (Table 1). As Table 1 shows, the $\alpha E284C$ mutation is the reason for this discrepancy, as the $\gamma Q274C$ and $\gamma L276C$ mutations by themselves did not significantly impair ATP synthase activity. We could exclude assembly problems or oligomeric instability as cause of the impairment (Table 1). Furthermore, the $\alpha E284C$ mutation did not cause uncoupling between ATP hydrolysis and proton translocation; uncoupling would have resulted in high membrane ATPase activity, but reduced ATP-driven proton pumping [25,63]. A number of deletion mutants in the DELSEED-loop have been described which combine high activity of the isolated F_1 with impaired ATP-driven proton pumping and decreased ATP synthesis rates as holoenzyme in membrane preparations [64–66]. The reason for this impairment seems to be that shortening of the DELSEED-loop reduces the rotational torque by half [64]. However, these mutants show high membrane ATPase activity. A possible explanation for the behavior of the $\alpha E284C$ mutant could be a further reduction in torque, allowing the enzyme to rotate an isolated γ subunit at full speed, but not an $\gamma\epsilon c_{10}$ rotor subcomplex integrated in the membrane. It should be noted that there is no simple structural explanation for such a far-reaching consequence of a point mutation.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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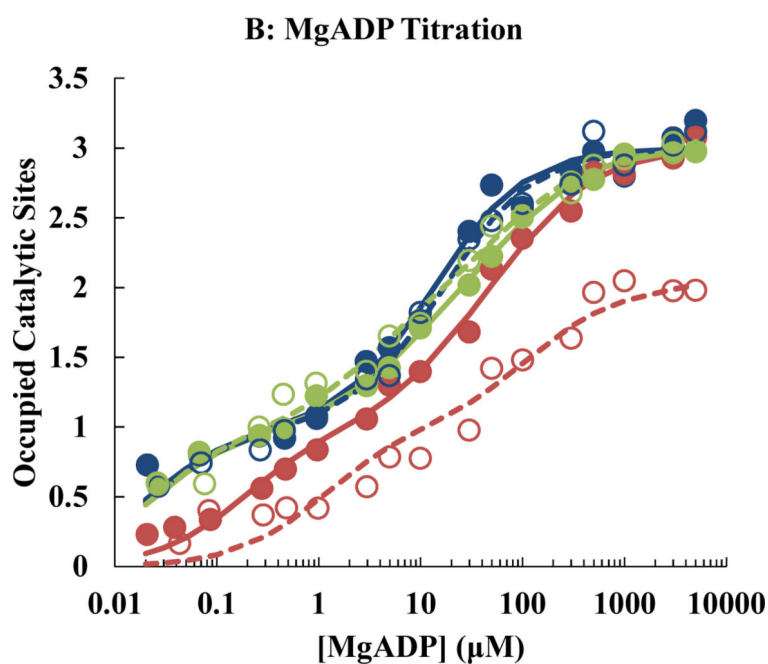
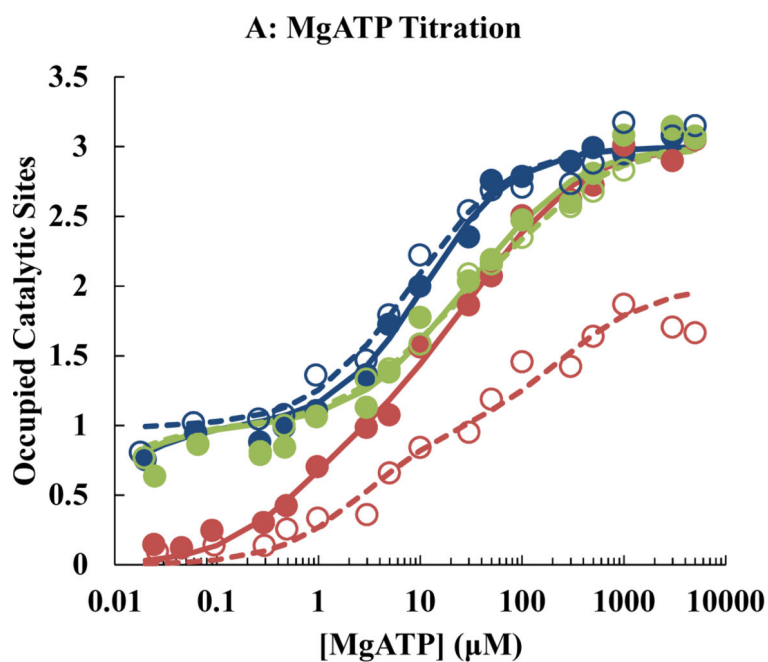
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Highlights

- The affinity of the catalytic sites of ATP synthase depends on the rotational angle of γ .
- Disulfide bonds lock the enzyme into the two most important conformations.
- Nucleotide binding affinities were measured at the catalytic and ATP-waiting dwell.
- Locking γ into the ATP-binding dwell results in the “loss” of a catalytic site.
- Our observations support the tri-site catalytic mechanism of ATP synthase in *E. coli*.



C: MgADP-AlF₄ Titration

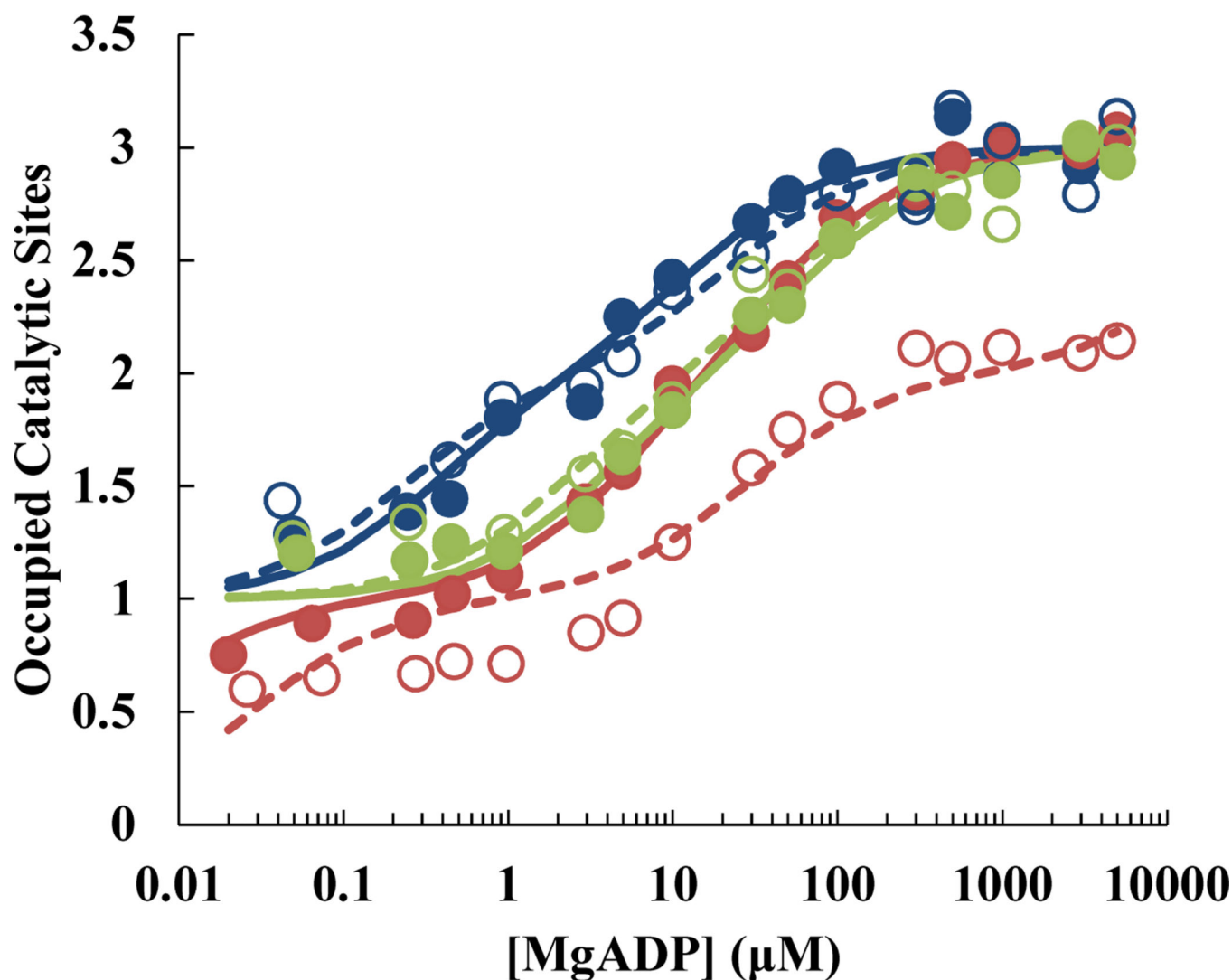


Fig. 1.

Titration of WT and mutant F₁ with nucleotides. Data for WT are shown in blue, those for the α E284C/ γ Q274C mutant in red, and those for the α E284C/ γ L276C mutant in green. Filled circles represent the experimental data of F₁ in absence of α - γ crosslink; open circles represent the experimental data of F₁ in presence of α - γ crosslink. Each data point represents the average of at least three independent assays. Solid lines are the least-squares best fit curves of the filled circle sets, whereas dashed lines are the least-squares best fit curves of the open circle sets. The curves were fitted as described in Materials and Methods, using a linear x-axis. Conversion to a log scale is only for illustration purposes, to allow presentation of binding events with vastly different affinities in a single figure.

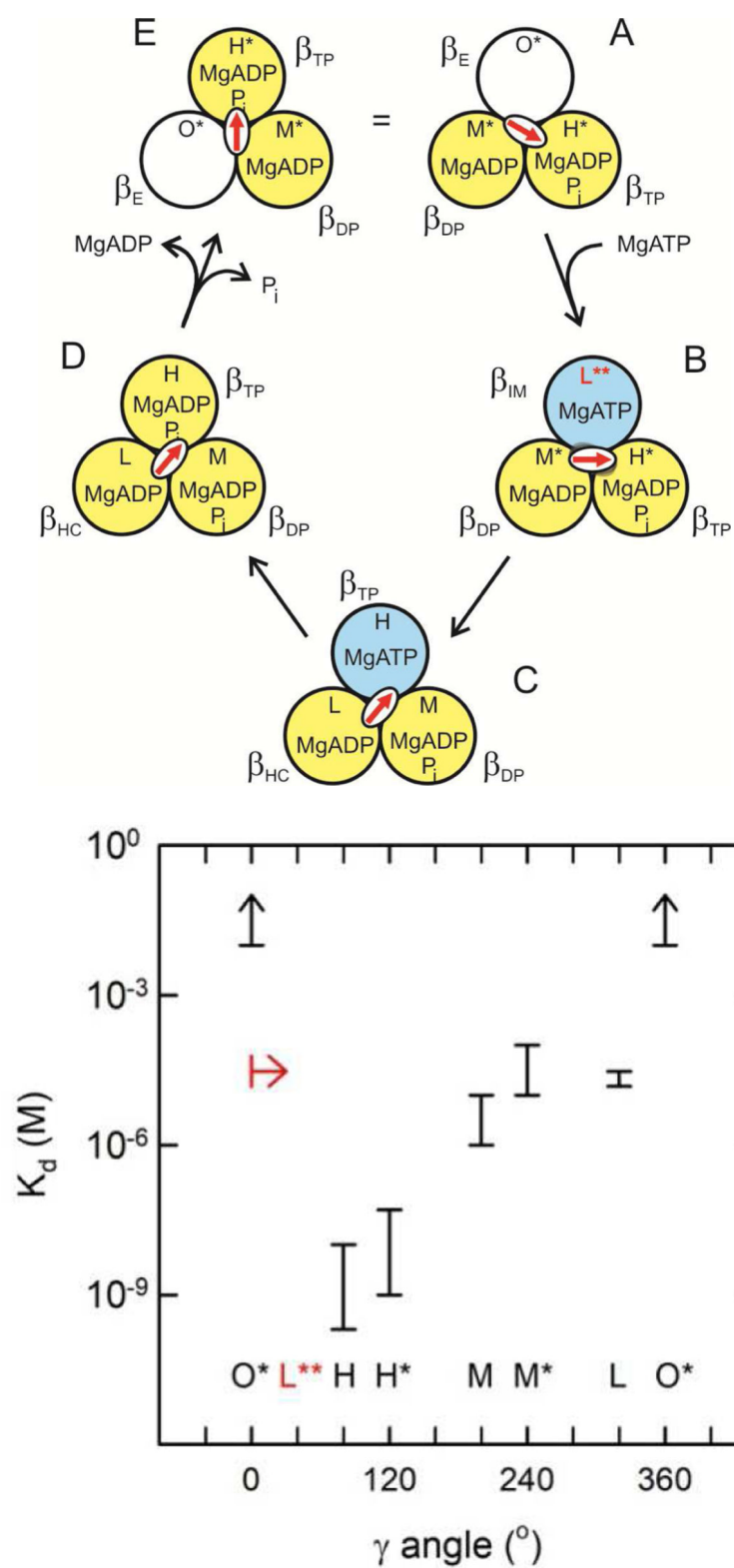


Fig. 2.

Catalytic site affinity changes in the rotary mechanism of ATP synthase. (A) Revised catalytic mechanism [15] incorporating the results of the present study. For details, see part 4.2. of the Discussion. It should be noted that the red arrow symbolizes the changes in the rotational angle of γ ; it is not associated with a specific structural feature of γ . The hypothetical intermediate conformation β_{IM} during closing of β might or might not be the same as the half-closed conformation β_{HC} seen in crystal structures [14,41]. (B) Catalytic site affinity as a function of the rotational angle of γ . For each specified angle, the approximate range of experimentally-determined affinities is given (for details, see part 4.2. of the Discussion). The letters indicate the symbols for the different affinities of the catalytic sites used in Fig. 2A (H, high; M, medium; L, low; O, open site; asterisk, ATP-waiting dwell; no asterisk, catalytic dwell). The red horizontal arrow and the red L** indicate that γ probably has to undergo thermal rotational fluctuations in the positive direction before MgATP can bind effectively (see part 4.4. of the Discussion; see also state B in Fig. 2A).

Table 1

Functional characterization of mutants used in the present study

Strain	Growth Yield		ATP Synthase Amount	ATPase Activity			ATP-driven H ⁺ Pumping
	8 mM Succinate	4 mM Glucose					
	%	%	%	unit/mg protein			%
				Reduced	Oxidized	Ox/Red	
WT (βY331W)	100	100	100	1.05 (0.04)	1.01 (0.03)	0.99 (0.03)	100
+ αE284C	68 (4)	91 (3)	137 (21)	0.17 (0.02)	0.14 (0.01)	0.15 (0.02)	45 (3)
+ γQ274C	104 (4)	97 (2)	122 (17)	0.80 (0.05)	0.74 (0.04)	0.78 (0.04)	97 (2)
+ γL276C	102 (3)	99 (2)	117 (15)	0.79 (0.03)	0.72 (0.04)	0.75 (0.05)	102 (3)
+ αE284C/ γQ274C	62 (3)	85 (4)	113 (12)	0.13 (0.02)	0.05 (0.01)	0.12 (0.02)	26 (4)
+ αE284C/ γL276C	72 (4)	88 (3)	115 (17)	0.12 (0.02)	0.04 (0.01)	0.12 (0.02)	42 (4)
pUC18	< 1	29 (1)	0	< 0.01	< 0.01	< 0.01	< 1

All strains generated in this study are based on pSN6/DK8 background (labeled “WT”) and carried the βY331W mutation. Growth yield in limiting succinate was assessed from the turbidity of cell culture, measured as absorbance at 590 nm. Inverted membrane vesicles isolated from WT and mutant strains were used for the ATPase activity and ATP driven H⁺ pumping measurements. The amount of membrane-bound ATP synthase was determined by Western blot using anti-γ antibody. Before enzymatic assays, membrane protein samples were treated with 1 mM DTT (the Reduced group), 1 mM CuSO₄ (the Oxidized group) at room temperature for 1 h in order to reduce or form a disulfide bond. Samples in the Ox/Red group were treated with 1 mM CuSO₄ for 1 h and then 4 mM DTT for 1 h at room temperature. This group was served to clarify whether the formation of disulfide bond is reversible. ATPase activities were quantified by the amount of inorganic phosphate released at 30 °C; 1 unit is defined as 1 μmol inorganic phosphate released per minute in the reaction. ATP-driven proton pumping ability was assayed as described in Materials and Methods. All values were normalized against WT, except for the membrane ATPase activity. Standard deviations are shown in parentheses. Strain pUC18/DK8 served as negative control, expressing no ATP synthase.

Table 2ATPase activities of purified F₁ samples

F ₁ Samples	No Treatment	1 mM DTT	1 mM CuSO ₄
	unit/mg protein		
WT (βY331W)	6.0 (0.4)	5.9 (0.2)	5.7 (0.4)
+ αE284C/γQ274C	5.1 (0.3)	5.0 (0.3)	0.2 (< 0.1)
+ αE284C/γL276C	4.0 (0.2)	3.9 (0.2)	0.1 (< 0.1)

F₁ samples were treated for 1 h with either 1 mM DTT or 1 mM CuSO₄ to reduce or oxidize the disulfide bond between α and γ subunits. All treatments were done at 25 °C. Experimental procedure of ATPase activity assay was as described in the legend of Table 1. Standard deviations are shown in parentheses.

Table 3Nucleotide dissociation constants of WT and mutant F₁

	WT (βY331W)			+ αE284C/γQ274C			+ αE284C/γL276C		
MgATP (μM)	K_{d1}	K_{d2}	K_{d3}	K_{d1}	K_{d2}	K_{d3}	K_{d1}	K_{d2}	K_{d3}
Reduction	< 0.01	6.0 (2.5)	34 (17)	0.66 (0.17)	15 (5.5)	93 (44)	< 0.01	10 (3.5)	81 (35)
	$R^2 = 0.9614$			$R^2 = 0.9755$			$R^2 = 0.9554$		
Oxidation	< 0.01	7.6 (2.9)	58 (28)	3.0 (0.68)	432 (105)	> 5000 [#]	< 0.01	8.0 (2.2)	140 (56)
	$R^2 = 0.9628$			$R^2 = 0.9207$			$R^2 = 0.9580$		
MgADP (μM)	K_{d1}	K_{d2}	K_{d3}	K_{d1}	K_{d2}	K_{d3}	K_{d1}	K_{d2}	K_{d3}
Reduction	0.022 (0.0049)	11 (12)	16 (19)	0.20 (0.041)	19 (5.7)	120 (47)	0.025 (0.0035)	7.3 (1.1)	88 (18)
	$R^2 = 0.9843$			$R^2 = 0.9859$			$R^2 = 0.9891$		
Oxidation	0.024 (0.0050)	12 (7.5)	24 (16)	1.3 (0.34)	160 (42)	> 5000 [#]	0.026 (0.0057)	3.6 (0.84)	76 (25)
	$R^2 = 0.9854$			$R^2 = 0.9408$			$R^2 = 0.9713$		
MgADP-AlF ₄ (μM)	K_{d1}	K_{d2}	K_{d3}	K_{d1}	K_{d2}	K_{d3}	K_{d1}	K_{d2}	K_{d3}
Reduction	< 0.01	0.39 (0.13)	12 (4.9)	< 0.01	5.7 (1.2)	42 (11)	< 0.01	3.9 (0.98)	89 (30)
	$R^2 = 0.9289$			$R^2 = 0.9874$			$R^2 = 0.9665$		
Oxidation	< 0.01	0.22 (0.072)	27 (11)	0.035 (0.010)	36 (9.9)	> 5000 [#]	< 0.01	2.6 (0.82)	83 (36)
	$R^2 = 0.9278$			$R^2 = 0.9207$			$R^2 = 0.9375$		

Nucleotide binding to the catalytic sites was measured at 25 °C by monitoring the fluorescence signal of residue βY331W. Nucleotide dissociation constants (K_d) were calculated by fitting theoretical curves to the experimental data by using a three-site binding model. The coefficient of determination (R^2) for each regression is listed. The method applied was unable to resolve K_d values lower than 0.01 μM with confidence. In the underlying titrations, each data point was measured at least three times. The least-square fits to determine binding constants and statistical parameters were based on these individual data points (not on the average of each condition shown in Fig. 1). Standard errors for the K_d values are shown in parentheses.

[#]This number was too high to be determined through current experimental method ($K_{d3} > 5$ mM).