Alteration of the Nucleotide-binding Site Symmetry of Chloroplast Coupling Factor 1 by Catalysis*

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Fluorescence resonance energy transfer was used to show that ATP hydrolysis induces a change in the properties of two nucleotide-binding sites in isolated chloroplast coupling factor 1 (CF1). The fluorescence donor was Lucifer Yellow vinyl sulfone (4-amino-N-[3-(vinylsulfonly)phenyl]napthalimide-3,6-disulfonate), covalently bound to a unique site on the α subunit between nucleotide-binding sites 2 and 3. The fluorescence acceptor was the ATP analog 2′(3′)-trinitrophenyladenosine 5′-triphosphate (TNP-ATP), incorporated specifically into nucleotide-binding site 1.

Energy transfer from Lucifer Yellow to TNP-ATP in site 1 was greater if catalysis occurred before TNP-ATP was incorporated than if no catalysis occurred, indicating that one of the nucleotide-binding sites near the Lucifer Yellow had changed its properties to those of site 1 as a result of catalysis. The amount of energy transfer increased with the degree of substrate excess during catalysis, as expected if catalysis were required for the new site 1 location. ADP, which binds to CF1, but is not a substrate for hydrolysis, caused little energy transfer. Titration of site 3 with TNP-ATP showed greater energy transfer from Lucifer Yellow when catalysis had not occurred, indicating that sites 1 and 3 switched properties as a result of catalysis.

The amount of energy transfer declined exponentially with time between removal of substrate and addition of TNP-ATP to site 1, with a half-time of 1.5–2 h at room temperature. This result suggests that the change that results in switching of nucleotide-binding sites 1 and 3 relaxes in the absence of substrate.

Our results show that the asymmetry of the nucleotide-binding sites of CF1 is not a permanent feature of the molecule.

Chloroplast coupling factor 1 (CF1),† the catalytic portion of the ATP synthase of thylakoid membranes, converts the energy of a transmembrane proton gradient into the chemical energy of ATP. The entire complex consists of eight or nine types of polypeptides. CF1 is a peripheral membrane protein attached to the membrane-embedded component Fo, which translocates protons across the membrane. CF1 can be detached from the membrane and studied in solution.

CF1 consists of five types of polypeptides, called α through ε in order of decreasing molecular weight. The polypeptide stoichiometry of CF1 is α3β2εδ (1), and the molecular weight is 400,000 (2). The α and β subunits are arranged with pseudohexagonal symmetry (3), alternating around a ring (4). The smaller γ, δ, and ε subunits are located between the membrane and the α and β subunits (5–7). Because the smaller subunits cannot participate in the apparent 3-fold symmetry of the α and β subunits, the molecule must be asymmetric overall. This structural asymmetry likely gives rise to some important functional asymmetries.

CF1 has three well-defined adenine nucleotide-binding sites associated with the β subunits (8–10), but the sites have different properties (9). Site 1 contains tightly bound ADP when CF1 is isolated. This ADP will exchange with medium nucleotides but does not dissociate in their absence. Site 2 binds MgATP with high affinity and does not exchange even after extensive catalytic turnover. Site 3 binds nucleotides in the presence or absence of divalent cations and is readily exchangeable. Site 3 is likely to be a catalytic site, and site 1 is probably also catalytic (11). The function of site 2 is unknown.

The catalytic mechanism of CF1 remains largely a mystery. One problem is the nature of the involvement of multiple nucleotide-binding sites in catalysis. It has been suggested that each catalytically active nucleotide-binding site participates in only a portion of the full catalytic cycle during each catalytic event and that the different sites exchange properties with each event. In one such "alternating site catalytic cooperativity" or "binding change" mechanism (12), product release at one catalytic site is triggered by substrate binding at a second site. The sites would then reverse roles for the next round of catalysis.

This type of model requires that the properties of the catalytic sites change, or alternate, during catalysis. These changes presumably would be evident as a change in the relative locations of the nucleotide-binding sites on CF1, with respect to a fixed point. The sites themselves would not necessarily move, only their properties. It should be possible then to detect the change by comparing the locations of the nucleotide sites before and after catalysis.

Lucifer Yellow vinyl sulfone (4-amino-N-[3-(vinylsulfonly)phenyl]napthalimide-3,6-disulfonate) is a fluorescent probe which, as a result of the asymmetry of the CF1 molecule, labels lysyl 378 in one of the three chemically identical α subunits of CF1 (13). It can be used as a fluorescence donor in fluorescence resonance energy transfer distance measure-
ments in which 2'-(3')-trinitrophenyladenosine 5'-triphosphate (TNP-ATP) in one of the nucleotide-binding sites acts as the fluorescence acceptor. The Lucifer Yellow molecule serves as the fluorescence donor, and the distance between the nucleotide-binding sites to be determined.

In this paper, we describe experiments which show that the configuration of nucleotide-binding sites 1 and 3 of isolated CF, is randomized when the enzyme catalyzes ATP hydrolysis.

MATERIALS AND METHODS

Thylakoid membranes were prepared from 1-2 kg of market spinach (14), CF, was isolated as described previously (15-17), with modifications (18). The enzyme was greater than 95% pure as judged by sodium dodecyl sulfate gel electrophoresis, with the only noticeable contaminant being ribulose-bisphosphate carboxylase/oxygenase. CF, was stored as an ammonium sulfate precipitate at 4 °C. The protein was deaerated prior to use by passage through 3-ml Sephadex G-50 (fine) centrifuge columns (19). All desalting steps were carried out by this procedure, hereafter referred to as "centrifuge columns." Protein concentration was determined by a modification of the method of Lowry et al. (20) using bovine serum albumin as a standard.

Modification of CF, with Lucifer Yellow vinyl sulfone (Aldrich) was performed in 50 mM Bicine-NaOH (pH 9.0), 3 mM MgCl, (15). CF, (10-100 μM) was incubated at room temperature with 50-100 μM Lucifer Yellow for 20 min, exchanged, and excess Lucifer Yellow was removed by passage through a centrifuge column equilibrated with 50 mM Tris-HCl (pH 8.0), 50 mM NaCl, 2 mM EDTA. All subsequent manipulations were carried out in this buffer. The stoichiometry of Lucifer Yellow labeling was determined using an extinction coefficient for bound TNP-ATP of 2.51 X 10^4 M^-1 cm^-1 at 425 nm after correcting for light scattering due to CF, (21). Stoichiometries were kept low (below about 0.5 mol of Lucifer Yellow/mol of CF,) to reduce nonspecific labeling. Neither ATPase activity nor nucleotide binding by CF, is significantly altered by labeling with Lucifer Yellow (13).

Reduction of the disulfide bond of the subunit of CF, which activates the latent ATPase activity was performed in 50 mM dithiothreitol at room temperature for up to 14 h, with or without 5 mM ATP. Excess dithiothreitol was removed by passage through one or two consecutive centrifuge columns. The reduced disulfide bond was prevented from reoxidizing by reaction with 5 mM N-ethylmaleimide for 5-10 min at room temperature. Excess N-ethylmaleimide was removed by passage through an additional centrifuge column. ATP hydrolysis was determined by colorimetric measurement of Pi release (23).

In some experiments, activated CF, was next incubated with 20 mM ATP and 5 mM MgCl, at 37 °C for 5-10 min. This procedure was intended to fill nucleotide-binding sites to reduce subsequent incorporation of TNP-ATP into this site in samples not exposed to ATP and CaCl, before TNP-ATP incorporation into site 1. At least 6 h were allowed to elapse after removal of excess ATP.

AATPase activity was carried out with 5-100 mM ATP and 10 mM CaCl, at 37 °C for 5-10 min. Substrates, products, and CaCl, were removed by passage through two or three centrifuge columns. Control samples contained no ATP and 0 or 10 mM CaCl, ADP and ATP were compared at a concentration of 50 mM, with 10 mM CaCl,.

Specific labeling of nucleotide-binding site 1 was performed essentially as described previously (13). Lucifer Yellow-labeled CF, was incubated with 100 μM TNP-ATP (Molecular Probes, Inc.), which is a strong inhibitor of CF,ATPase activity (24), for 2 h at room temperature. Unbound and dissociable TNP-ATP were removed by two successive column centrifugations. Labeling stoichiometries were determined using an extinction coefficient for bound TNP-ATP of 2.51 X 10^4 M^-1 cm^-1 at 418 nm (25), after subtracting the absorbance at 418 nm due to Lucifer Yellow and light scattering by CF, This correction was determined from a Lucifer Yellow-labeled CF, sample to which no TNP-ATP was bound. Stoichiometries of 0.9-1.1 mol of TNP-ATP/mol of CF, were obtained. Stoichiometries greater than 1.0, which sometimes occurred in samples not treated with ATP + CaCl, were assumed to be due to incorporation of TNP-ATP into nucleotide-binding site 2. Since this phenomenon would have obscured the observed effects rather than accentuated them, no correction was made for it.

Absorption spectra were obtained with a Beckman DU-7 spectrophotometer. Fluorescence measurements were made on a Farrand

801 spectrophotometer. The excitation and emission wavelengths were 440 and 525 nm, respectively, for Lucifer Yellow. CF, was diluted to approximately 0.5 μM for fluorescence measurements. Adjustments were made for the small protein concentration differences between samples. All samples within an experiment had identical Lucifer Yellow/CF, stoichiometries because samples were taken as aliquots from a single labeling reaction.

Site 3 labeling was performed by titrating Lucifer Yellow-labeled CF, already containing TNP-ATP in site 1 with up to 50 μM TNP-ATP in the fluorometer cuvette (6). The dissociation constant for TNP-ATP binding to site 3 is approximately 4 μM (9). Equilibrium was reached after each addition within a few seconds. Energy transfer between bound Lucifer Yellow and free TNP-ATP was negligible.

For titrations of nucleotide-binding site 3 of CF, with TNP-ATP, corrections were made for sample dilution due to TNP-ATP additions, for TNP-ATP fluorescence, and for the inner filter effect due to TNP-ATP. The fluorescence due to TNP-ATP in solution was determined by titration of buffer in the fluorometer. Changes due to the presence of 0.5 μM CF, were negligible. The inner filter effect coefficients for each concentration of TNP-ATP were determined by titrating free Lucifer Yellow at submicromolar concentration with TNP-ATP. After subtracting the TNP-ATP fluorescence, the Lucifer Yellow fluorescence expected if it were reduced only by dilution was divided by the observed fluorescence to obtain the coefficient for the inner filter effect at each TNP-ATP concentration.

An additional correction was made for fluorescence due to Lucifer Yellow bound to contaminating ribulose-bisphosphate carboxylase/oxygenase, which sometimes occurred in samples not treated with ATP (13). In other words, there is a correction for it.

Quantitation of labeling specificity was determined by high pressure liquid chromatography (HPLC) of complete trypsin digest of labeled CF, (12). Up to 100 μg of trypsin-digested CF, (100 μg) were injected onto an UltraspHERE ODS 4.6 mm x 25-cm reverse-phase C, column (Altex) attached to a Beckman 342 gradient liquid chromatograph equipped with a Model 157 fluorescence detector and a Spectra-Physics SP4270 integrator. Peptides were eluted with an acetonitrile gradient containing 0.1% trifluoroacetic acid, and the area of each fluorescent peptide peak was integrated. The labeled lysine 378-containing pentapeptide of the α subunit gave the only large fluorescent peak, amounting to 65-85% of the total fluorescence detected, depending on the extent of labeling and contamination of CF, with ribulose-bisphosphate carboxylase/oxygenase. The labeled peptide was purified on a 5-mm microcolumn containing a solid matrix synthesized by the method of Lowry al. (20). The pentapeptide was characterized by amino acid analysis and by the method of Libby's cyanogen bromide cleavage of the tryptic peptide and comparison with the sequence of the α subunit. The α subunit was digested with cyanogen bromide (19). N-terminal sequencing was carried out by the dithiothreitol method of Edman (22). Sequence analysis was performed on an Applied Biosystems model 470A sequencer, and the results were interpreted with a model 120 computer software package.
3 switch during catalysis, then after a random number of rounds of ATP hydrolysis by a population of CF\textsubscript{i} molecules, all of which have undergone catalysis, half will be in the "switched" conformation and half will be in the original conformation after the substrate is removed (Fig. 1). This is the basis for the experiments described here.

We will not discuss a model in which sites 1–3 alternate during catalysis because it is unlikely that site 2 participates in catalysis. Our analysis could be altered by the participation of additional catalytic sites, however (see "Discussion").

The latent ATPase activity of Lucifer Yellow-labeled CF\textsubscript{i} was activated by reduction of the \( \gamma \) subunit disulfide bond. Reoxidation of the bond was prevented by alkylation of the sulphydryls with N-ethylmaleimide. The sample was divided in half, and one-half was allowed to hydrolyze CaATP. The other half was incubated in the absence of substrate. High concentrations of ATP (up to 100 mM) were used because of high CF\textsubscript{i} concentration. Site 1 was then labeled with TNP-ATP in both samples. Aliquots of the samples were diluted to about 0.5 \( \mu \)M CF\textsubscript{i}, and the Lucifer Yellow fluorescence was measured. Fluorescence is expressed as relative fluorescence units/micromolar CF\textsubscript{i}.

In 25 determinations, exposure of the enzyme to CaATP caused a decrease in Lucifer Yellow fluorescence relative to the control of 6.6 to 22.3% (mean = 11.0%, median = 11.1%, and S.D. = 3.8%). This decrease was not due to loss of the Lucifer Yellow label since no significant label stoichiometry differences were detected between samples that had and had not been exposed to CaATP. The fluorescence quenching is a result of energy transfer from Lucifer Yellow to TNP-ATP since it is reversed by displacement of TNP-ATP with ATP (data not shown). Similar results were obtained when MgATP replaced CaATP. Fluorescence decreases below the theoretical maximum of 20% indicate that fewer than half of the CF\textsubscript{i} molecules had nucleotide sites 1 and 3 switched after ATP hydrolysis, due in part to an insufficient excess of ATP substrate over enzyme. Additionally, the apparent amount of energy transfer is reduced by contamination of CF\textsubscript{i} with a small amount of ribulose-bisphosphate carboxylase/oxygenase, which labels strongly with Lucifer Yellow, but whose fluorescence is unaffected by TNP-ATP.

The efficiency of fluorescence energy transfer is independent of the stoichiometry of the fluorescence donor, as long as each protein molecule has no more than one donor molecule. This is because only fluorescent molecules are observed; unlabeled ones are invisible. The Lucifer Yellow/CF\textsubscript{i} stoichiometry was changed by varying the labeling time. Fig. 2 shows that there was no significant effect of label stoichiometry on the amount of fluorescence reduction caused by ATP hydrolysis.

Our experimental model (Fig. 1) predicts that the amount of fluorescence reduction in the sample that hydrolyzed ATP is proportional to the fraction of CF\textsubscript{i} molecules having site 1 proximal to the Lucifer Yellow label when TNP-ATP is added. This prediction is testable by varying that fraction between 0 and 0.5.

A direct way to vary the amount of catalysis by a CF\textsubscript{i} sample is to vary the molar ratio of substrate ATP to CF\textsubscript{i}, during catalysis (Fig. 3). When the ATP/CF\textsubscript{i} ratio was kept low, the amount of energy transfer was small because few CF\textsubscript{i} molecules had an opportunity to hydrolyze ATP during the 5-min incubation due to a lack of substrate as well as inhibition by product ADP. As the ATP/CF\textsubscript{i} ratio was increased, more CF\textsubscript{i} molecules were able to hydrolyze ATP, and the amount of energy transfer increased. At the highest ATP/CF\textsubscript{i} ratio used, which required 0.3 mM ATP due to the high enzyme concentration, the fluorescence decrease due to energy transfer approached, but did not reach, the theoretical maximum.

ADP inhibits ATP hydrolysis by CF\textsubscript{i} (26). Although CaADP can bind to the active site of CF\textsubscript{i}, it caused very little energy transfer. In three experiments, 50 mM ATP and 10 mM CaCl\textsubscript{2} caused 12.4 ± 1.9% energy transfer (mean ± S.D.), whereas 50 mM ADP and 10 mM CaCl\textsubscript{2} caused only 2.5 ± 0.8%, a value close to the error level of the experiment.

The concentration of ATP needed to achieve maximal energy transfer is far greater than that needed to saturate completely the nucleotide-binding sites with ATP (K\textsubscript{a} for site 3 is about 4 \( \mu \)M (9)). Moreover, TNP-ATP binding to the nucleotide-binding sites does not in itself cause the nucleotide sites to switch properties, or else both ATP-treated and -untreated samples would show the same amount of energy.

![Figure 1](image1.png)

**Fig. 1.** Model for outcome of experiment in which TNP-ATP is specifically incorporated into nucleotide-binding site 1 of CF\textsubscript{i}, which has (B) or has not (A) catalyzed ATP hydrolysis, assuming a two-site catalytic mechanism involving randomization of sites 1 and 3. The nucleotide-binding sites are located on the \( \beta \) subunits. The asterisk indicates the \( \alpha \) subunit susceptible to labeling with Lucifer Yellow. LY, Lucifer Yellow covalently bound to CF\textsubscript{i}; ATP, substrate for catalysis, including divalent cations; TNP, TNP-ATP, which is noncovalently bound to CF\textsubscript{i}.

![Figure 2](image2.png)

**Fig. 2.** Effect of Lucifer Yellow/CF\textsubscript{i} labeling stoichiometry on amount of fluorescence decrease in CF\textsubscript{i} to which TNP-ATP has been added to nucleotide-binding site 1 after ATP hydrolysis. \( \%F \), percent decrease in fluorescence of the sample that has hydrolyzed ATP subtracted from that of the sample that has not. Fluorescence measurements were made on five aliquots of each sample. LY, Lucifer Yellow.
transfer. These observations, in combination with the lack of ADP-induced energy transfer, suggest that ATP hydrolysis, rather than substrate binding, is required for the nucleotide-binding sites to switch their properties.

Although these experiments supported the hypothesis that the catalytic sites randomize during ATP hydrolysis, a serious theoretical problem remained. The energy transfer mapping of distances between several discrete sites on CF1 showed that all isolated CF1 molecules have the same conformation (for a recent review, see Ref. 27). The Lucifer Yellow site is always on the α subunit between nucleotide-binding sites 2 and 3. Our experiments, on the other hand, showed that after ATP hydrolysis there are two populations of CF1 molecules, one with site 1 and the other with site 3 near the Lucifer Yellow label. Therefore, when CF1 is isolated from thylakoids, it should have two conformational populations, and the energy transfer mapping would not have yielded sensible results.

The problem can be reconciled if, in the absence of added nucleotides or when stored as an ammonium sulfate precipitate, CF1 "relaxes" to a preferred conformation in which nucleotide-binding site 3 is proximal to Lucifer Yellow and site 1 is distal. Relaxation would have to be fairly slow. If all of the switched nucleotide sites were to relax rapidly, no energy transfer would have been detected.

This hypothesis was confirmed by experiment. As the time between removal of substrate and addition of TNP-ATP was increased, the amount of energy transfer decayed in a roughly exponential manner (Fig. 4). The half-time of relaxation at room temperature was approximately 1.5–2 h. Relaxation of switched nucleotide sites during the approximately 20–30 min between ATP removal and TNP-ATP addition partly accounts for the submaximal energy transfer observed when even quite high ATP/CF ratios were used during ATP hydrolysis.

Exposure of the relaxed enzyme to CaATP before addition of TNP-ATP restored energy transfer between Lucifer Yellow and TNP-ATP in nucleotide-binding site 1. Twenty to thirty minutes were required to remove the substrate and add the TNP-ATP. Data are means ± S.D. for four (0.5, 2.5, 4.5 h), three (1.5, 3.5, 5.5 h), or two (20 h) determinations.

No loss of energy transfer occurred within 3 h after TNP-ATP was incorporated into site 1. If both sites 1 and 3 are completely filled with TNP-ATP, then the fluorescence of a sample which catalyzed ATP hydrolysis should be identical to the fluorescence of one that did not. This can be seen by inspection of Fig. 1. If both sites 1 and 3 were filled, half of the sample should show the fluorescence of one that did not catalyze ATP hydrolysis.
Nucleotide Site Switching in CF1

and 3 contain TNP-ATP on all CF1 molecules, there is an equal amount of fluorescence energy transfer between TNP-ATP and Lucifer Yellow, regardless of whether the nucleotide sites are in the "preferred" or switched positions.

Site 3 is titrated by adding increasing amounts of TNP-ATP to Lucifer Yellow-labeled CF1, having site 1 prefilled with TNP-ATP. Lucifer Yellow fluorescence should decrease as the TNP-ATP concentration increases because energy transfer to TNP-ATP in site 3 increases as more TNP-ATP is bound. The sample that hydrolyzed ATP should start with lower fluorescence than the sample that did not because of energy transfer between Lucifer Yellow and TNP-ATP in proximal site 1. As site 3 is filled, however, the fluorescence of the sample that did not hydrolyze ATP should decrease faster than the fluorescence of the sample that did because the former has more site 3 proximal to the Lucifer Yellow label than the latter. This prediction was confirmed by experiment (Fig. 6).

TNP-ATP/CF1 stoichiometries in excess of 1.0 in the sample that did not hydrolyze ATP, which was probably due to labeling of nucleotide-binding site 2 emptied of MgATP during storage in EDTA-containing buffer (31), reduced the magnitude of the convergence in the early part of the titration. Nevertheless, the results clearly support the hypothesis.

DISCUSSION

Our results clearly show that nucleotide-binding site 1 assumes the properties of site 3, and vice versa, as a result of ATP treatment of soluble CF1 and suggest that ATP hydrolysis is involved. Thus, the nucleotide-binding site asymmetry, previously described for CF1 (see Ref. 27), is not a permanent feature of the enzyme. The consequences of these observations for understanding the mechanism of ATP hydrolysis or synthesis by the enzyme, however, remain unclear. Our observations are consistent with both the alternating site, or binding change, mechanism (12) and with a mechanism in which the nucleotide-binding sites become equivalent during catalysis, but do not necessarily alternate. Although the binding change mechanism has received broad experimental support (29-32), other mechanisms are consistent with those and our data. Thus, although our data may be readily interpreted within the framework of the binding change mechanism, they should not be construed as supporting that hypothesis exclusively.

Our results are consistent with those of Melese and Boyer (32), who found that asymmetries in the covalent labeling of CF1 β subunits by dicyclohexylcarbodiimide and 2-azido-ATP were randomized as a result of hydrolysis of 2-azido-ATP.

A major concern is whether nucleotide-binding sites 1 and 3 are catalytic sites of the enzyme. There seems little doubt that site 1 can be catalytic since ADP bound to site 1 of CF1 in solution can be phosphorylated by medium P (33). Moreover, ATP in site 1 is slowly converted to ADP (29). The rate of nucleotide release or exchange from site 1 had been thought to be too slow to be consistent with the participation of site 1 in catalysis (25). Recently, however, it has been shown that under steady-state hydrolytic conditions, the rate of exchange of site 1 is approximately the same as the rate of catalysis (10), a result, incidentally, which supports our observations of the modification of the properties of site 1 by catalysis.

Mitochondrial and Escherichia coli F1 contain six nucleotide-binding sites (see Ref. 34); yet, only three have been described in detail for CF1, so far. Evidence for additional low affinity sites has been presented, however (35). Although these nucleotide-binding sites have not been characterized, it is possible that they are involved in the switching of the properties of sites 1 and 3 that we have detected. In addition, these low affinity sites could be involved in catalysis, either indirectly as regulatory sites or directly as catalytic sites (36). The presence of additional nucleotide-binding sites other than the three well-characterized ones does not affect our conclusion that the nucleotide site asymmetry is altered by catalysis. Whether this phenomenon is an essential aspect of the catalytic mechanism would, however, be more difficult to establish if additional sites are present. Alterations of the properties of site 1 have been implicated in the activation of membrane-bound CF1 (see Ref. 37), and it is conceivable that our results are as applicable to the mechanism of activation as they are to the mechanism of catalysis.

Nucleotide-binding sites 1 and 3 that have switched their properties as a result of catalysis slowly return to their original conformation after substrate is removed from the medium. This phenomenon has not been described before, although there was a hint of it in the experiments of Melese and Boyer (32). Perhaps one conformational state is thermodynamically slightly more stable as a result of the asymmetric interactions between the nucleotide-binding sites and the γ, δ, and ε subunits of the enzyme (7). Alternatively, relaxation could be a direct consequence of a catalytic or regulatory process operating in the absence of medium nucleotides.

We have shown that CF1, even in solution, has a dynamic structure, one that depends upon its prior history. Catalysis induces changes in the enzyme, and it will be of interest to map these changes through the use of fluorescence resonance energy transfer methods. The slow relaxation of the enzyme after exposure to Mg- or Ca-ATP may provide a means for studying hydrolysis-induced conformational changes in the

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enzyme and the relevance of these changes to the mechanism of ATP hydrolysis.

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