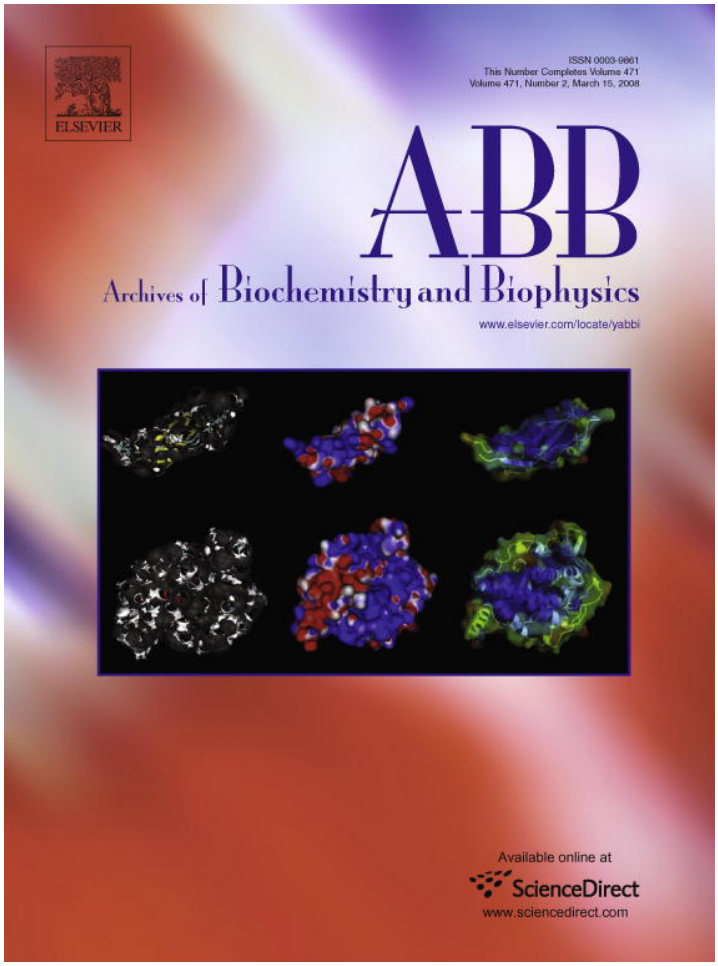

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# Role of αPhe-291 residue in the phosphate-binding subdomain of catalytic sites of *Escherichia coli* ATP synthase

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Received 24 November 2007, and in revised form 15 January 2008
Available online 26 January 2008

**Abstract**

The role of αPhe-291 residue in phosphate binding by *Escherichia coli* $F_1F_0$-ATP synthase was examined. X-ray structures of bovine mitochondrial enzyme suggest that this residue resides in close proximity to the conserved βR246 residue. Herein, we show that mutations αF291D and αF291E in *E. coli* reduce the ATPase activity of $F_1F_0$ membranes by 350-fold. Yet, significant oxidative phosphorylation activity is retained. In contrast to wild-type, ATPase activities of mutants were not inhibited by MgADP-azide, MgADP-fluoroaluminate, or MgADP-fluoroscandium. Whereas, 7-chloro-4-nitrobenzo-2-oxa-1,3-diazole (NBD-Cl) inhibited wild-type ATPase essentially completely, ATPase in mutants was inhibited maximally by ~75%, although reaction still occurred at residue βTyr-297, proximal to αPhe-291 in the phosphate-binding pocket. Inhibition characteristics supported the conclusion that NBD-Cl reacts in βE (empty) catalytic sites, as shown previously by X-ray structure analysis. Phosphate protected against NBD-Cl inhibition in wild-type but not in mutants. In addition, our data suggest that the interaction of αPhe-291 with phosphate during ATP hydrolysis or synthesis may be distinct.


*Keywords:* ATP synthesis; Oxidative phosphorylation; $F_1F_0$-ATP synthase; $F_1$-ATPase; Catalytic sites; Pi-binding assays; Nucleotide binding; Biological nanomotor

$F_1F_0$-ATP synthase is the smallest known biological nanomotor, found in species ranging from bacteria to man and is responsible for ATP synthesis by oxidative or photophosphorylation in membranes of bacteria, mitochondria, and chloroplasts. Thus, ATP synthase is the fundamental means of cellular energy production in animals, plants, and almost all microorganisms. A typical 70 kg human with relatively sedentary lifestyle will generate around 2.0 million kg of ATP from ADP and Pi in a 75-year lifespan [1].

*Escherichia coli* $F_1F_0$-ATP synthase contains eight different subunits within its soluble cytoplasmic $F_1$ sector (subunits $\alpha_3\beta_3\gamma\delta\varepsilon$) and its membrane-associated $F_0$ sector (subunits $ab_2c_{10}$). X-ray structures of bovine enzyme [2] established the presence of three catalytic sites at α/β subunit interfaces of $\alpha_3\beta_3$ hexamer in $F_1$ sector. Reactions at the catalytic sites are controlled by rotation of the γ subunit within the $\alpha_3\beta_3$ hexagon. The γ subunit is part of the "rotor" which is composed of γ, ε, and a ring of c subunits. The "stator" is composed of $b_2\delta$. The function of the stator is to prevent co-rotation of catalytic sites, as well as the a subunit, with the rotor. Detailed reviews of ATP synthase structure and function may be found in references [3–8].

The reaction mechanism of ATP synthesis and hydrolysis and their relationship to mechanical rotation is not fully understood. Recently, we have focused our efforts on determining how Pi binding is achieved. Understanding how Pi binding occurs is important for two reasons. First, it was shown [9–11] that Pi binding appears to be "energy

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doi:10.1016/j.abb.2008.01.013

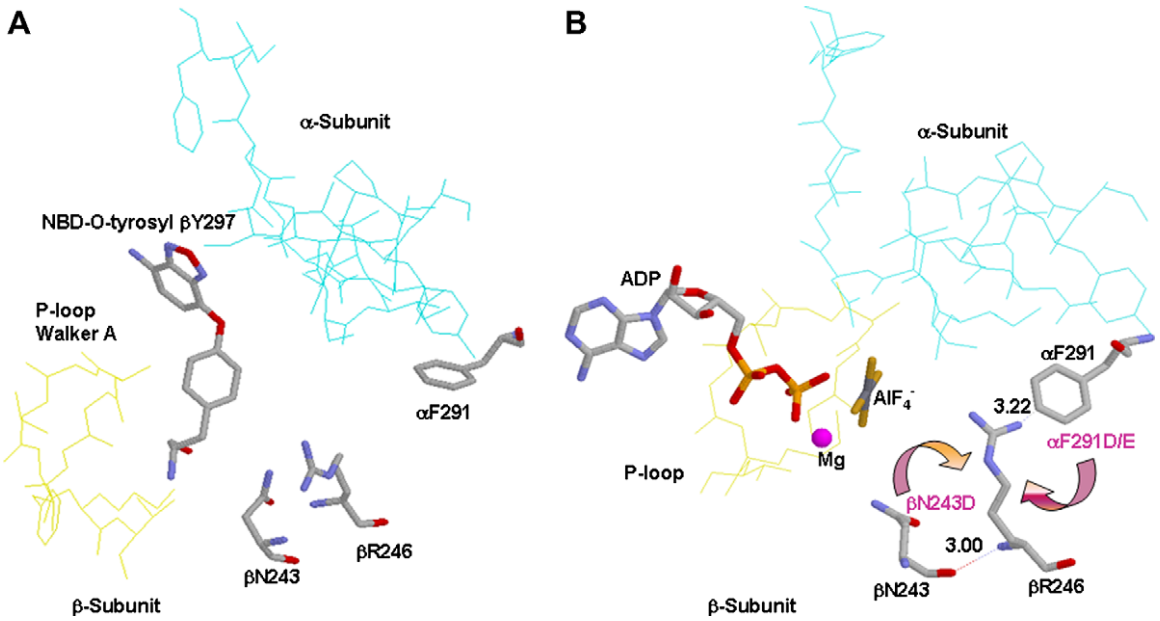


Fig. 1. X-ray structures of catalytic sites in mitochondrial ATP synthase showing spatial relationship of αF291 with βR246 and βN243. Rasmol software was used to generate these figures. (A) Reacted NBD-O-tyrosyl by297 in the βE site [14]. (B) The βDP site in the $AlF_4^-$-inhibited enzyme [17]. *E. coli* residue numbering is shown.

linked", and directly to subunit rotation. Second, as an explanation of how ATP synthase binds ADP and Pi within its catalytic sites in the face of a relatively high concentration of ATP/ADP concentration ratio. In an effort to resolve this problem, Weber and Senior [12] proposed that rotation-linked binding of Pi occurs as a first step. Next, this would allow ADP binding and sterically prevent ATP binding. Due to the lack of any direct experimental approach the progress on determination on the Pi binding residues was hindered. Thus, we turned to an assay devised by Perez et al. [13]. In this assay, Pi binding is indicated by protection from inhibition of ATPase activity induced by covalent interaction with 7-chloro-4-nitrobenzo-2-oxa-1,3-diazole (NBD-Cl)[1]. Since Orris et al. [14] showed by X-ray crystallography that the covalent adduct formed by NBD-Cl is specifically in the βE catalytic site (see Fig. 1A) protection afforded by Pi indicates that binding of Pi occurs at the βE catalytic site. Using this system as a Pi binding assay, we were able to identify four Pi binding residues in the *E. coli* enzyme. For details see ref. [15]. It may be noted that Penefsky [16] recently detected $[^{32}P]P_i$ binding with a $K_d(P_i)$ in the range of 0.1 mM using an alternative, pressure ultra filtration method, and this result is consistent with data obtained from the NBD-Cl protection assay. It is apparent that Pi dissociates more rapidly from *E. coli* $F_1$ than it does from mitochondrial $F_1$, unfortunately, rendering the convenient centrifuge assay inapplicable with the *E. coli* enzyme.

After binding, Pi must be condensed with MgADP via a chemical transition state. A molecular mechanism for this process was proposed by Senior et al. [1]. Fig. 1B shows the transition state analog $MgADP{\cdot}AlF_4^-$ trapped in catalytic site of the mitochondrial enzyme as determined by X-ray crystallography [17]. It is clear from the geometry of this complex that the fluoroaluminate group occupies the position of phosphate in the predicted transition state. Similarly, Ko et al. [18] reported the first transition state-like structure of $F_1$ using enzyme obtained from rat liver and crystallized with the phosphate (Pi) analog vanadate (Vi). In this case the structure was useful since it was consistent with biochemical data obtained earlier by Pedersen et al. [19] whereby inhibition by Vi indicated a transition state-like complex. However, this case was particularly interesting since they demonstrated that ADP was not essential suggesting that $MgVi–F_1$ complex inhibited the catalytic activity to the same extent as that observed for the $MgADP{\cdot}Vi–F_1$ complex. Unfortunately, MgVi or MgADP·Vi does not inhibit the *E. coli* enzyme [20]. Thus, we have relied on inhibition of ATPase activity by fluoroaluminate (or fluoroscandium) in order to asses the potential to stabilize a transition state complex [15,20–22]. Through mutagenesis, and by employing the NBD-Cl protection assay, as well as ATPase inhibition by transition state analogs, we can investigate the involvement of potential residues that are critical for Pi binding. In this manuscript, we explore the possible role played by αPhe-291 residue. Fig. 1B shows the location of αF291 residue in the catalytic subdomain along with other identified residues. The basic question we asked was: does replacement of αPhe-291 with a negatively charged residue (either Asp or Glu) affect Pi binding?

[1] *Abbreviations used:* NBD-Cl, 7-chloro-4-nitrobenzo-2-oxa-1,3-diazole; DTT, dithiothreitol.

## Materials and methods

### *Preparation of E. coli membranes; measurement of growth yield in limiting glucose medium; assay of ATPase activity of membranes; measurement of proton pumping in membrane vesicles*

*Escherichia coli* membranes were prepared as in [23]. It should be noted that this procedure involves three washes of the initial membrane pellets. The first wash is performed in buffer containing 50 mM TES, pH 7.0, 15% glycerol, 40 mM 6-aminohexanoic acid, 5 mM *p*-aminobenzamidine. The following two washes are performed in buffer containing 5 mM TES, pH 7.0, 15% glycerol, 40 mM 6-aminohexanoic acid, 5 mM *p*-aminobenzamidine, 0.5 mM DTT, 0.5 mM EDTA. Prior to the experiments, membranes were washed twice more by resuspension and ultracentrifugation in 50 mM $TrisSO_4$, pH 8.0, 2.5 mM $MgSO_4$. Growth yield in limiting glucose was measured as in [24]. ATPase activity was measured in 1 ml assay buffer containing 10 mM NaATP, 4 mM $MgCl_2$, 50 mM $TrisSO_4$, pH 8.5, at 37 °C. Reactions were started by addition of membranes and stopped by addition of SDS to 3.3% final concentration. Pi released was assayed as in [25]. For wild-type membranes (10–20 μg protein), reaction times were 2–10 min. For mutant membranes (50–100 μg protein), reaction times were 30–60 min. All reactions were shown to be linear with time and protein concentration. ATP-driven proton pumping was measured by following the quench of acridine orange fluorescence as described in [26]. SDS–gel electrophoresis on 10% acrylamide gels was as in [27]. Immunoblotting with rabbit polyclonal anti-$F_1$-α and anti-$F_1$-β antibodies was as in [28].

### *Construction of wild-type and mutant strains of E. coli*

Wild-type strain was pBWU13.4/DK8 [29]. Mutagenesis was by the method of Vandeyar et al. [30]. The template for oligonucleotide-directed mutagenesis was M13mp18 containing the Hind3–Xba1 fragment from pSN6. pSN6 is a plasmid containing the βY331W mutation from plasmid pSWM4 [31] introduced on a Sac1–Eag1 fragment into pBWU13.4 [29] which expresses all the ATP synthase genes. Mutagenic oligonucleotide for αF291D[2] was: CGGGCGACGTCGACTACCTCCACTCTCG where the underlined bases introduce the mutation and a new Sal1 restriction site; αF291E, CGGGCGACGTCGAATACCTCCACTCTCG where the underlined bases introduce the mutation and a new AatII restriction site. DNA sequencing was performed to confirm the presence of mutations and absence of undesired changes in sequence, and the mutations were transferred to pSN6 on an Xho1–Pml1 fragment generating the new plasmids pZA11 (αF291D/βY331W) and pZA12 (αF291E/βY331W). Each plasmid was transformed into strain DK8 [32] containing a deletion of ATP synthase genes for expression of the mutant enzymes. It may be noted that all of the new mutant strains contained the βY331W mutation, which is valuable for measurement of nucleotide binding parameters [31] and does not affect function significantly. While it was not utilized in this work, the Trp mutation was included for possible future use.

### *Inhibition of ATPase activity by NBD-Cl and protection by MgADP or Pi*

NBD-Cl was prepared as a stock solution in dimethyl sulfoxide and protected from light. Membranes (0.2–2.0 mg/ml) were reacted with NBD-Cl for 60 min in the dark, at room temperature, in 50 mM $TrisSO_4$, pH 8.0, 2.5 mM $MgSO_4$, then 50 μl aliquots were transferred to 1 ml of ATPase assay buffer to determine ATPase activity. Where protection from NBD-Cl inhibition by ADP or Pi was determined, membranes were preincubated 60 min with protecting agent at room temperature before addition of NBD-Cl. $MgSO_4$ was present, equimolar with ADP or Pi. Control samples containing the ligand without added NBD-Cl were included. Neither Pi (up to 50 mM) nor MgADP (up to 10 mM) had any inhibitory effect alone.

### *Reversal of NBD-Cl inhibited ATPase activity by DTT*

Where reversal of NBD-Cl inhibition by DTT was measured, membranes were first reacted with NBD-Cl (150 μM) for 1 h at room temperature, then DTT (final = 4 mM) was added and incubation continued for 1 h at room temperature before ATPase assay. Control samples without NBD-Cl and/or DTT were incubated for the same times.

### *Inhibition of ATPase activity by fluoroaluminate, fluoroscandium, or sodium azide*

Membranes were incubated for 60 min at room temperature in 50 mM $TrisSO_4$, 2.5 mM $MgSO_4$, 1 mM NaADP, and 10 mM NaF at a protein concentration of 0.2–1.0 mg/ml in presence of $AlCl_3$ or $ScCl_3$ added at varied concentration (see Results). Fifty microliter aliquots were then added to 1 ml of ATPase assay buffer and activity measured as above. It was confirmed in control experiments that no inhibition was seen if $MgSO_4$, NaADP, or NaF was omitted. For measurements of azide inhibition, membranes were preincubated with varied concentrations of sodium azide. Then 1 ml ATPase assay buffer was added to measure the activity.

## Results

### *Growth properties of αF291D and αF291E mutants of E. coli ATP synthase*

Two new mutants, αF291D and αF291E, were generated. This residue was chosen for mutagenesis due to its proximity to βArg-246. In earlier studies, βArg-246 was shown to be a key residue for binding of Pi since mutation of βArg-246 to Ala (βR246A) abrogated Pi binding as assessed by several assays (see Fig. 1 and ref. [20]). αF291D and αF291E mutants were designed with the intent that introduction of a negative charge may negate the nearby positive charge of βArg-246 and may also directly negatively affect Pi binding through electrostatic interactions. Previous mutagenesis data lead us to suspect that electrostatic interactions within the catalytic site are critical for activity. We found that the introduction of Asp at position βAsn-243 appears to nullify the positive charge of βArg-246 [21]. Reciprocally, introduction of a positive charge in the form of Arg at position αPhe-291 can compensate the loss of Arg by βR246A mutation [22].

Table 1 shows the growth yield on limiting glucose medium and growth on succinate plates. It is evident that introduction of Asp or Glu resulted in lower growth suggesting that oxidative phosphorylation is defective (yet operative) in both αF291D and αF291E. Specific ATPase and proton pumping activities of membrane preparations of mutant enzymes were compared with wild-type and null control and the values are shown in Table 1. It may be noted that the membrane preparations were washed extensively before assays. Duplicate or triplicate preparations of the same mutant membranes gave identical low specific activity. The conclusion is that the insertion of Asp or Glu close to the Pi binding residue βArg-246 reduced the ATPase

[2] *E. coli* residue numbers used throughout.

Table 1
Effects of α Phe-291 mutation on cell growth and ATPase activity

| Mutation[a] | Growth[b] on succinate | Growth yield in limiting glucose (%) | ATPase activity[c] (μmol/min/mg) | Proton pumping[d] (%) |
|---|---|---|---|---|
| Wild-type | ++++ | 100 | 28 | 84 |
| Null | – | 46 | 0 | 0 |
| αF291D | ++ | 70 | 0.07 | 0 |
| αF291E | ++ | 72 | 0.09 | 0 |

[a] Wild-type, pBWU13.4/DK8; Null, pUC118/DK8. All mutants were expressed with the βY331W mutation also present, which does not significantly affect growth. Data are means of four to six experiments each.
[b] Growth on succinate plates after 3 days estimated by eye. ++++, heavy growth; ++, light growth; –, no growth.
[c] Measured at 37 °C and expressed as μmol ATP hydrolyzed/min/mg membrane protein. Each individual experimental point is itself the mean of duplicate assay tubes. Data are derived from two separate membrane preparations. Results from separate membrane preparations were in excellent agreement.
[d] Measured using acridine orange and expressed as percent quench of acridine orange fluorescence in membrane vesicles upon addition of 1 mM MgATP.

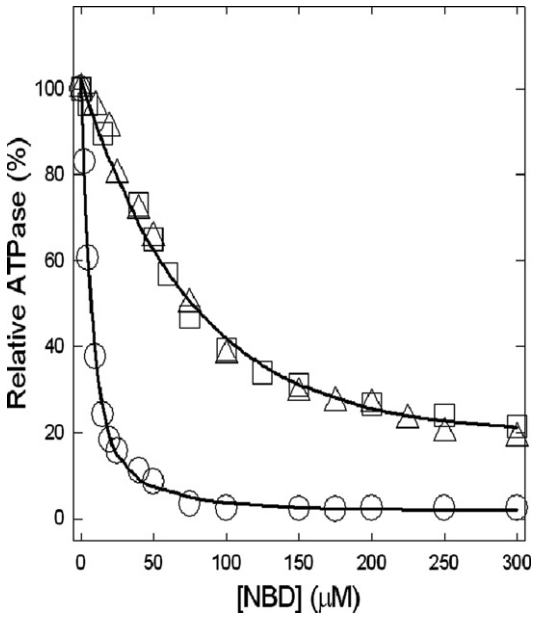


Fig. 2. Inhibition of ATPase activity in membrane-bound wild-type and mutant ATP synthase by NBD-Cl. Membranes were preincubated for 60 min at 23 °C with varied concentration of NBD-Cl, then aliquots added to 1 ml of assay buffer and ATPase activity determined. Details are given in Materials and methods. Symbols used are: ○, wild-type; □, αF291D; △, αF291E. Each data point represents average of at least four experiments, using two independent membrane preparations of each mutant. Results agreed within ±10%.

activity to a very low level. Moreover, ATPase activities of the mutants were far too low to support proton pumping. Membranes prepared from the mutants contained the same amount of α and β subunits as wild-type; therefore, reduced ATPase is not due to loss of $F_1$. Previously, we established that Pi binding by mutant and wild-type ATP synthase can be assayed using either membrane preparations or purified $F_1$ [20,22]. Coomassie blue-stained SDS–gel electrophoresis, immunoblotting and densitometry of mutants and wild-type membranes (with purified wild-type $F_1$ as reference) established the purity and integrity of $F_1$-α and $F_1$-β subunits [22,28].

*Inhibition of ATPase activity of ATP synthase in membranes by NBD-Cl and reversal by dithiothreitol*

Fig. 2 shows NBD-Cl inhibition of wild-type and mutant membranes in the presence of varied concentrations of NBD-Cl. In wild-type, potent inhibition occurred and this is consistent with previous studies [15,20–22,33,34]. However, the mutants were inhibited approximately 75–80%. In previous studies [15,20–22,33], we have noted several instances where mutant ATP synthase were incompletely inhibited by NBD-Cl. To be sure that the maximal reaction with NBD-Cl had been reached, we incubated each membrane preparation with 150 μM NBD-Cl for 1 h as in Fig. 2 followed by an additional pulse of 200 μM NBD-Cl and continued the incubation for an additional hour before assaying ATPase activity. Very little or no additional inhibition occurred (Fig. 3A) consistent with Fig. 2 data. This shows that the reaction of NBD-Cl was complete and fully reacted membranes retained residual activity. Next, we wanted to check if inactivation by NBD-Cl could be reversed by addition of the reducing agent DTT since reversibility by DTT is indicative of specificity of crosslinking in previous studies. When wild-type and mutant enzymes were preincubated with 125 μM NDBD-Cl as in Fig. 2 and then 4 mM DTT was added and incubation continued for 1 h before ATPase assay, it was seen that DTT completely restored the full activity in all cases (Fig. 3B). This indicates that NBD-Cl reacts specifically with residue βTyr-297 in the mutants as well as in wild-type [35,36].

*Protection against NBD-Cl inhibition of ATPase activity by MgADP or Pi*

We previously found that MgADP protects against NBD-Cl inhibition of wild-type soluble $F_1$ as well as membrane preparations of $F_1F_0$; however, protection occurred only at high concentrations ($EC_{50} \sim 4.5$ mM). We reasoned that high concentrations were required in order to effectively keep the third site βE occupied in time average and thus impede the access to NBD-Cl by sterically obstructing the site [15,20–22,33]. This idea would be consistent with the conclusion of Orris et al. [14] who provided evidence that NBD-Cl reacts specifically in the βE catalytic site by X-ray crystallographic studies. Fig. 4 shows the data for MgADP protection against NBD-Cl in membrane enzymes. Wild-type and both mutants were protected against NBD-Cl inhibition, with similar $EC_{50}$ values of about 4.5 mM. From this we conclude that NBD-Cl is reacting in βE site in the mutants, and that the activities

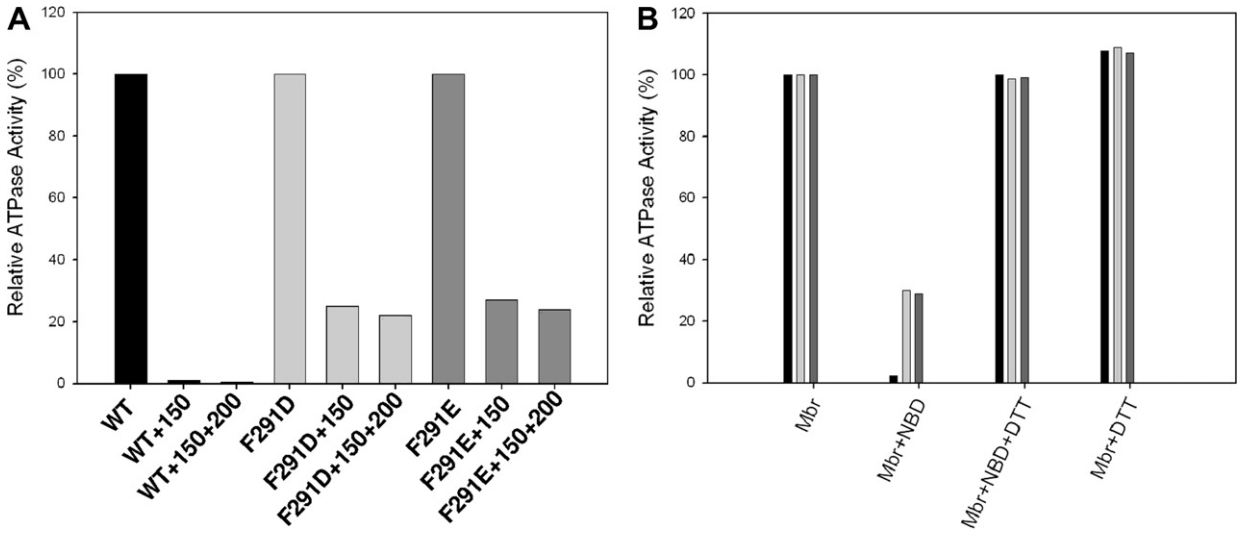


Fig. 3. Results of extra pulse of NBD-Cl in mutants and reversal of NBD-Cl inhibition by DTT. (A) Membrane ATP synthase (Mbr) was inhibited with 150 μM NBD-Cl for 60 min under conditions as described in Fig. 1. Then a further pulse of 200 μM NBD-Cl was added and incubation continued for 1 h before assay. (B) Membrane ATP synthase (Mbr) was incubated with or without 150 μM NBD-Cl for 60 min under conditions as described in Fig. 1. Degree of inhibition was assayed. In parallel samples, 4 mM DTT was then added, and incubation continued for further 60 min before assay. Each bar graph represents wild-type, αF291D, and αF291E from left to right.

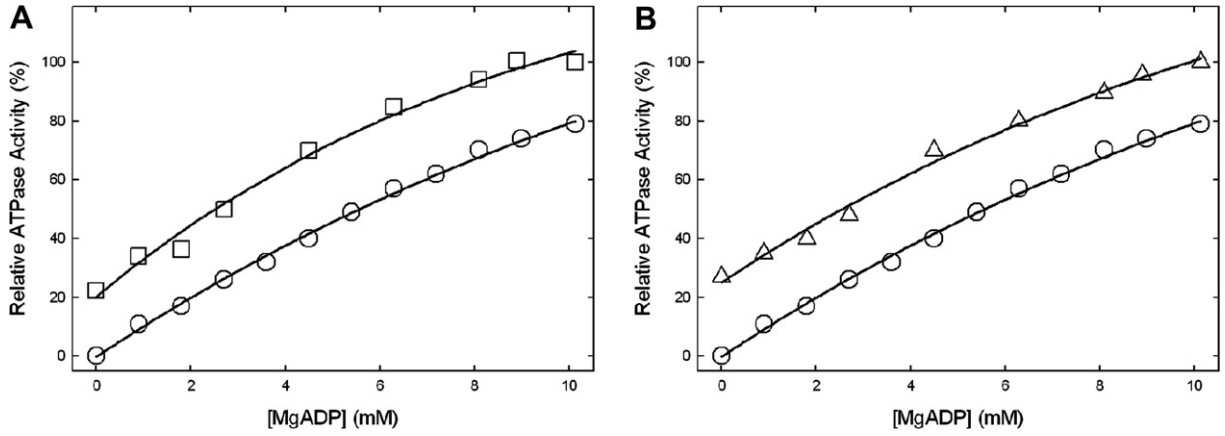


Fig. 4. Protection against NBD-Cl reaction by MgADP. Wild-type and mutant membrane were preincubated for 1 h at room temperature with varied concentration of MgADP as shown, then 125 μM NBD-Cl was added and incubation continued at room temperature in the dark for 1 h. Aliquots were then assayed for ATPase activity. Symbols used are: ○, wild-type; □, αF291D; △, αF291E. Results are means of quadruplicate experiments which agreed within ±10%.

measured are due to ATP synthase enzyme and not due to a contaminant.

Fig. 5 presents the data on protection against NBD-Cl reaction by Pi. In this case, Pi protected well against NBD-Cl inhibition of ATPase activity in wild-type preparations but did not protect against inactivation in αF291D or αF291E mutants.

### *Inhibition of ATPase activity by sodium azide, fluoroaluminate, and fluoroscandium in membranes containing mutant ATP synthase*

Since the mutants appeared defective in Pi binding as assessed by protection from NBD-Cl inhibition by Pi itself, we next chose to examine phosphate analogs. Consistent with earlier studies, Fig. 6A shows wild-type was completely inhibited. Yet, the mutants, αF291D or αF291E, were remarkably resistant to inhibition by sodium azide. Azide is a potent inhibitor of ATPase. Recent X-ray crystallographic studies indicate that azide forms a MgADP-$N_3$ complex in which azide is located in the same position as the gamma phosphate of ATP [37]. Fig. 6B and C show the inhibition of wild-type and mutant enzymes by fluoroaluminate and fluoroscandium, respectively. Also consistent with earlier studies, wild-type was strongly-inhibited both by fluoroaluminate (>95%) and by fluoroscandium (>98%). Yet, as with azide, in the presence of fluoroaluminate and fluoroscandium, the mutants fail to show any sign of inhibition. It should be noted that both mutants showed the same

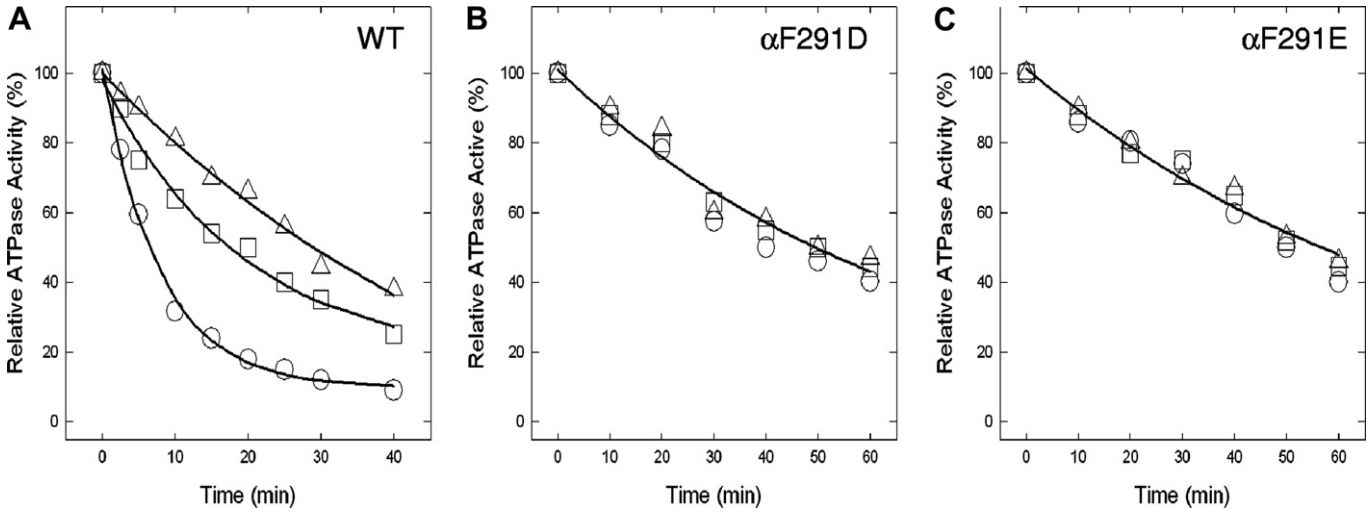


Fig. 5. Protection by Pi of ATPase activity in wild-type and mutant membranes from inactivation by NBD-Cl. Membranes were preincubated with Pi at 0, 2.5 or 10 mM concentration as shown, for 60 min at 23 °C. Then NBD-Cl (125 μM) was added and aliquots withdrawn for assay at time intervals as shown. ATPase activity remaining is plotted against time of incubation with NBD-Cl. ○, no Pi added; □, 2.5 mM Pi; △, 10 mM Pi. Each data point represents the average of four different experiments using two independent membrane preparations of each mutant.

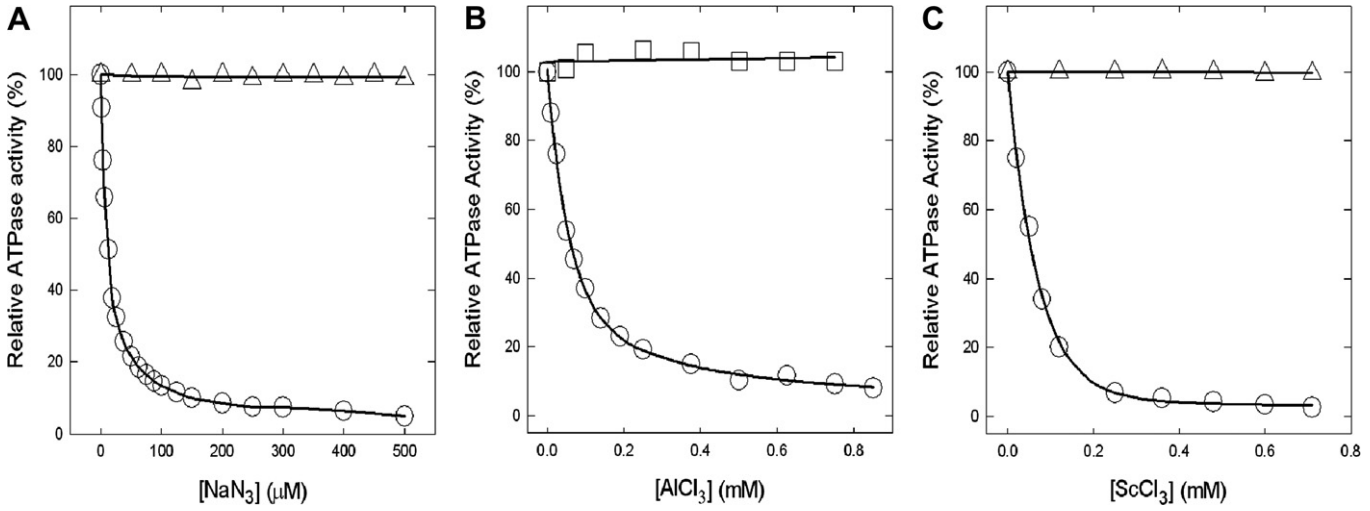


Fig. 6. Inhibition of membrane ATPase activity from mutant and wild-type ATP synthase enzymes by azide, fluoroaluminate, and fluoroscandium. Sodium azide was added directly to the membranes and incubated for 30 min before assay (A). Membranes were preincubated for 60 min at 23 °C with 1 mM MgADP, 10 mM NaF and indicated concentration of $AlCl_3$ (B), or $ScCl_3$ (C) then aliquots were added to 1 ml of assay buffer and ATPase activity determined. For details see Experimental procedures. The symbols used are: ○, wild-type; □, αF291D; △, αF291E, mutant. To maintain clarity data points for one or the other mutants are shown. All the data points are means of at least quadruplicate experiments. Variation was ±10% between different experiments.

behavior in all three assays, although for the purpose of clarity only one is shown in each panel.

## Discussion

The goal of this study was to examine the functional role(s) of residue αPhe-291 of *E. coli* ATP synthase. Residue αPhe-291 is located at the α/β interface flanking the Pi binding pocket (Fig. 1B). The X-ray crystal structures of the $AlF_3$-inhibited enzyme [38] as well as the $AlF_4$-inhibited enzyme which also contained $SO_4$ [17] show that the side chain of residue αPhe-291 points towards these bound Pi analogs. Pi binding is a primary step in ATP synthesis by ATP synthase thus understanding the molecular basis of Pi binding is an important way to examine and understand the functional role of residues in the catalytic site.

βArg-246 is one of four residues that have already been shown to be critical for Pi binding [15,20,33]. αPhe-291 resides at a suitable distance (3.2 Å) from βArg-246 in the $AlF_3$ containing catalytic site and 7.5 Å in the $SO_4$ containing catalytic site. Our earlier studies demonstrated that modulation of charge in the Pi binding site by way of mutagenesis could be a helpful tool in deciphering the molecular mechanism of Pi binding as well as the functional roles of

residues within the catalytic site(s) [21,22]. In that early work, we established the use of the Pi protection assay (against NBD-Cl inhibition) as well as the use of phosphate analogs in order to confirm the role of residues suspected to be involved in Pi binding [20–22,33]. As an example of the effectiveness of mutagenesis when combined with this assay, we demonstrated that introduction of negative charge at position βAsn-243 in the form of Asp resulted in the loss of Pi binding [21]. Using similar techniques, we also found that introduction of a positive charge (βN243R or αF291R) compensated for the loss of Pi binding by βR246A [22]. Given the proximity of αPhe-291 to the Pi binding pocket in available structures it appeared to be a suitable location for the introduction of negative charge. We chose to make two mutations αF291D and αF291E. Both of the mutants (αF291D or αF291E) failed to show any Pi binding (Fig. 5). Moreover, several other assays including loss of inhibition by azide, fluoroaluminate, and fluoroscandium all point to the fact that Pi binding in the mutants is abrogated.

Given our data, the question arises: what explanations may account for the loss of Pi binding by αF291D or αF291E mutant enzymes? One possibility is that the mutants may directly negatively affect Pi binding through electrostatic interactions. A second possibility is that they may indirectly affect Pi binding by neutralizing the positive charge of βArg-246. While we cannot exclude the first possibility, distance considerations appear to favor the latter argument.

Both mutations (αF291D or αF291E) rendered the preparations insensitive to azide. In the structure of bovine $F_1$-ATPase determined at 1.95-Å resolution with crystals grown in the presence of ADP, 5′-adenylyl-imidodiphosphate, and azide, Bowler et al. [37] showed that the azide anion interacts with the β-phosphate of ADP residing in the ADP-binding catalytic subunit, βDP. It occupies a position similar to the site occupied by the gamma-phosphate in the ATP-binding subunit, βTP. Its presence in the βDP-subunit appears to tighten the binding of the side chains to the nucleotide, enhancing its affinity and thereby stabilizing the state with bound ADP. Therefore, inhibition of the wild-type enzyme presented in Fig. 6A is to be expected. It has been suggested earlier [39] that βArg-246 is responsible for conformational signaling between catalytic sites required for rapid catalysis. If signaling is defunct in the mutants enzymes, with resultant low ATPase, then further inhibition by azide is not expected. This infers to the neutralization of βArg-246 positive charge by the introduction of negative charge in the vicinity.

MgADP.vanadate complex is an inhibitory transition state analog that has been used to study a variety of ATPase enzyme [18,19,40,41]. Unfortunately, both wild-type and mutant *E. coli* $F_1$ proved to be resistant to inhibition by vanadate [20]. Fluoroaluminate and fluoroscandium in combination with ADP and $Mg^{2+}$ ions potently inhibits wild-type or βY331W *E. coli* ATP synthase [20–22,33,42,43]. Both are believed to mimic the chemical transition state. Transition state-like structures involving bound MgADP·$AlF_4^-$ complex have been seen in two catalytic sites by X-ray crystallography [17,38]. Given the dramatic differences seen with fluoroaluminate, our results suggest that αPhe-291 is involved in transition state stabilization. Alternatively, if the effect of αPhe-291 mutation occurs via neutralization of βArg-246, then our data further implicate βArg-246 in transition state stabilization.

Another noteworthy aspect of our data is that the mutants display unusual behavior with regard to succinate utilization. Growth assays using succinate as the sole carbon source indicate that a significant amount of oxidative phosphorylation is retained. This is curious since ATPase activity as well as Pi binding is highly defective. We do not have a simple explanation for this unexpected result. One exciting possibility is that the role of αPhe-291 in Pi binding is different during ATP synthesis (in the oxidative phosphorylation direction) versus during ATP hydrolysis. This implies that the parameters controlling Pi binding are not the same in the forward and backward reactions. A second explanation for our results is that, *in vivo*, a modicum of activity that may not be detected by our *in vitro* assays may be sufficient to preserve function and viability. While further experiments will be required to determine if mutation of αPhe-291 plays different roles during synthesis or hydrolysis, the studies presented herein provide further evidence of the importance of electrostatic interactions in the $F_1F_0$ Pi binding catalytic site.

## Acknowledgments

This work was partly supported by the ETSU Major RDC Grant 0061 and RS 0021 to Z.A. We are grateful to Dr. Alan Senior, Professor Emeritus, Department of Biochemistry & Biophysics, University of Rochester Medical Center, Rochester, NY, for his comments on the manuscript.

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