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Functional importance of α Ile-346 and α Ile-348 in the catalytic sites of *Escherichia coli* ATP synthase

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Abstract

We studied the functional role of highly conserved VISIT-DG sequence residues α Ile-346 and α Ile-348 in the catalytic sites of *Escherichia coli* F₁F₀ ATP synthase. α Ile-346 is in close proximity, 2.98 and 3.63 Å, to the two known phosphate binding residues α R376 and β R182; α Ile-348 is situated within 3.66 Å from β R182. Single or double mutants of both α I346 and α I348 resulted in a variable loss of oxidative phosphorylation and ATPase activity. Azide, fluoroaluminate, and fluoroscandium caused insignificant to significant inhibition of mutants. Whereas the wild-type enzyme was completely inhibited by NBD-Cl (7-chloro-4-nitrobenzo-2-oxa-1, 3-diazole), a variable extent of inhibition was observed for α I346 and α I348 mutants. MgPi protection against NBD-Cl induced inhibition of wild-type, α I346, and α I348 demonstrated that, although strongly conserved, α I346 and α I348 have no direct role in phosphate binding. Insertion of Arginine in the form of α I346R/ β R182A, α I346R/ α R376A, or α I348R/ β R182A was able to compensate for the absence of known phosphate-binding Arginine residues β R182 and α R376. Results also suggest that α Ile-346 and α Ile-348 seem to have functional importance in upholding the phosphate-binding subdomain and transition state stabilization in the catalytic sites of *E. coli* ATP synthase.

Keywords

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Keywords

F₁F₀ ATP synthase; *E. coli* ATP synthase; ATPase inhibition; oxidative phosphorylation; Pi binding residues

Introduction

F₁F₀ ATP synthase, the smallest known biological nanomotor, is the main source of ATP generation in almost all organisms. Structurally, *Escherichia coli* has the simplest form of ATP synthase with five subunits arranged in the water-soluble F₁ sector ($\alpha_3\beta_3\gamma\delta\epsilon$) and three subunits in the membrane-associated F₀ sector (ab_2c_{10}) [1, 2]. Structural and functional aspects of membrane bound F₁F₀ ATP synthase enzyme are highly conserved among different species. ATP hydrolysis and synthesis take place at the α/β interface, whereas proton transport occurs through the membrane-embedded F₀ sector [3, 4].

Catalytic and motor functions of ATP synthase are connected to the Pi (inorganic phosphate) binding residues and to residues surrounding the Pi binding subdomain in the catalytic sites [5]. MgPi protection against 7-chloro-4-nitrobenzo-2-oxa-1, 3,-diazole (NBD-Cl) induced inhibition of ATPase activity provides an indirect but useful method to measure Pi binding [6]. Because NBD-Cl covalently interacts with β 297 in the β E catalytic site [7], the protection by Pi specifies that Pi binds at the β E (empty) catalytic site. Modification of the Pi protection assay initially used in mitochondrial membranes for *E. coli*, purified F₁ or membrane bound F₁F₀, allowed us to previously investigate the role of nine residues, β Arg-246, β Asn-243, α Arg-376, β Lys-155, β Arg-182, α Phe-291, α Ser-347, Thr-349, and α Gly-351¹, in Pi binding. Four residues, β Arg-246, α Arg-376, β Lys-155, and β Arg-182, were directly involved in Pi binding by forming a triangle across Pi, and two residues, α Ser-347 and α Thr-349, were indirectly involved in Pi binding through their interaction with β Arg-246 and β Arg-182 (see Fig. 1) [5, 8–15]. Three residues, β Asn-243, α Phe-291, and α Gly-351, did not have a direct role in Pi binding even though all the above residues are positioned in close proximity to the phosphate analogs AlF₃ or SO₄²⁻ in X-ray structures of catalytic sites. [16, 17]. Fig.1A shows an SO₄²⁻ ion thought to mimic phosphate while in Fig.1B fluoroaluminate is in the position of the phosphate. α Ser-347, α Thr-349, and α Gly-351 are all part of the highly conserved VISIT-DG sequence. The VISIT-DG sequence is situated in close proximity to the α/β interface flanking the Pi binding subdomain in the catalytic sites (Fig. 1B).

Menz *et al* [17] determined the bovine ATP synthase X-ray crystallographic structure that showed the transition state analog MgADP·AlF₄⁻ trapped in catalytic sites (Fig. 1B). In this complex, the fluoroaluminate group occupies the position of the ATP- γ -phosphate in the predicted transition state. Senior *et al* [3] proposed the mechanism of Pi and MgADP condensation. Pedersen's group [18] reported the transition state-like structure of F₁ from rat liver crystallized with the Pi analog vanadate (V_i). The MgV_i-F₁ complex inhibited the catalytic activity to the same extent as the MgADP-V_i-F₁ complex, suggesting that ADP was

¹*E. coli* residue numbers used throughout.

not essential. In another study, we observed that neither purified F_1 nor membrane bound F_1F_0 from *E. coli* was inhibited by MgVi or MgADP-Vi [9]. Therefore, fluoroaluminate and fluoroscandium were used to assess the potential to stabilize the transition state complex [5, 8–11, 14, 15].

The first high-resolution crystal structure of *E. coli* F_1 sector was resolved by Cingolani and Duncan [19]. Resolution of the *E. coli* structure provides more opportunities for the development of effective antimicrobial drugs in comparison to mitochondrial ATP synthase [19, 20], such as the newly developed anti-tuberculosis drug bedaquiline that targets bacterial ATP synthase [21]. Unfortunately, the *E. coli* crystallographic structure [20] does not contain sulfate, phosphate, fluoroaluminate, fluoroscandium, or bound NBD-Cl making it unsuitable for the identification, characterization, and molecular modeling of Pi binding residues in the catalytic sites of *E. coli*. However, the mitochondrial ATP synthase structure with bound phosphate analogs and about 70% homology to the *E. coli* sequence can be used to study the Pi binding residues [16, 17, 22].

The well-preserved nature and contiguous location of VISIT-DG sequence residues with the Pi binding subdomain in the catalytic sites is indicative of their direct or indirect role in Pi binding and maintenance of the Pi binding subdomain. In the absence of direct Pi binding or release assay MgPi protection against NBD-Cl inhibition and the transition state analog induced inhibition profiles confidently allow us to investigate the direct or indirect role of residues in Pi binding. This manuscript describes the functional importance of two highly conserved α -subunit VISIT-DG sequence residues, α Ile-346 and α Ile-348, in Pi binding and their possibility of compensating for the other known Pi binding residues. Figure 1B shows the position of α Ile-346 and α Ile-348 residues with respect to other known Pi binding residues in the catalytic sites. The strongly conserved nature and proximity of α I346 and α I348 to the Pi binding subdomain residues resulted in the following study questions: What is the purpose of non-polar hydrophobic side chains of α I346 and α I348 in the polar Pi binding subdomain? Do α Ile-346 and α Ile-348 directly or indirectly participate in Pi binding? Will the mutations α I346A/D/Q/R or α I348A/D/Q/R have any effect on transition state formation? Moreover, will α I346R or α I348R be able to compensate for the nearby known Pi binding residues β Arg-246, β Arg-182, or α Arg-376?

Materials and Methods

E. coli wild-type and mutant strains

The wild-type *E. coli* strain used was pBWU13.4/DK8 [23]. Mutant strains were generated by the Vandeyar *et al* method [24]. Oligonucleotide-directed mutagenesis was performed using the M13mp18 template containing the HindIII-XbaI fragment from pSN6. Plasmid pSN6 expresses all the ATP synthase genes and has the β Y331W mutation from plasmid pSWM4 [25] introduced on a SacI-EagI fragment into pBWU13.4 [23]. Mutagenic oligonucleotides used in this study are listed in Table 1. The presence of correct mutations and absence of undesired changes in sequence was confirmed by DNA sequencing. All the mutations were transferred to pSN6 on a Csp451 (an isoschizomer of BstBI) and PmlI fragment, generating the new single plasmids pZA65(α I346A), pZA66(α I346D), pZA67(α I346Q), pZA68(α I346R), pZA69(α I348A), pZA70(α I348D), pZA71(α I348Q),

pZA72(α I348R), and pZA73(α R376A). Double mutants pZA74(α I346R/ α R182A), pZA75(α I346R/ α R376A), pZA76(α I346R/ β R246A), pZA77(α I348R/ α R182A), pZA78(α I348R/ α R376A), and pZA79 (α I348R/ β R246A) were generated by combining pZA65-pZA72 fragments on pZA24, pZA73, and pZA7 plasmids. All plasmids were transformed into the DK8 strain [26] from which ATP synthase genes were deleted for expression of the mutant enzymes. All mutant strains also contained the β Y331W mutation. This mutation is valuable tool for measuring nucleotide binding parameters [25] and has no significant effect on enzyme function. Although Trp mutation was not used in this study, the β Y331W mutation was included for possible future use.

Preparation of *E. coli* membranes, measurement of growth yield in limiting glucose medium, and assay of membrane bound F_1F_0 ATPase activity

E. coli membrane bound F_1F_0 ATP synthase were prepared by the Senior *et al* method [27]. Notably in this procedure, initial membrane bound F_1F_0 pellets are washed three times. The first wash is done in a buffer containing 50 mM TES pH 7.0, 15% glycerol, 40 mM 6-aminohexanoic acid, and 5 mM p-aminobenzamidine. The next two washes are carried out in a buffer containing 5 mM TES pH 7.0, 15% glycerol, 40 mM 6-aminohexanoic acid, 5 mM p-aminobenzamidine, 0.5 mM DTT, and 0.5 mM EDTA. Before the experiments, membranes are washed two additional times by resuspension and ultracentrifugation in 50 mM TrisSO₄ pH 8.0 and 2.5 mM MgSO₄. These extra washes are meant to reduce the null mutant to a true zero activity. Thus, low activities observed for mutants are their own and not that of any contaminants. Growth yield in limiting glucose was measured as described previously [28]. ATPase activity was measured in a 1 ml ATP assay buffer containing 10 mM NaATP, 4 mM MgCl₂, and 50 mM TrisSO₄ pH 8.5 at 37°C. Reactions were started by addition of membrane bound F_1F_0 and stopped by addition of 1 ml SDS to a final concentration of 3.3%. Taussky and Shorr reagent was used to measure the released Pi [29]. Reaction times for 20–30 μ g wild-type membrane bound F_1F_0 protein were 5–10 min, and reaction times for 40–60 μ g membrane bound F_1F_0 protein were 30–50 min. All reactions were found to be linear with respect to time and protein concentration. The purity of membrane bound F_1F_0 was checked by SDS-gel electrophoresis on 10% acrylamide gels and by immunoblotting with rabbit polyclonal anti- F_1 - α and anti- F_1 - β antibodies as described previously [30] and shown in Fig 2.

Inhibition of ATPase activity by NBD-Cl and protection by MgADP or MgPi

NBD-Cl stock solution was prepared in dimethyl sulfoxide (DMSO) and protected from light. Membrane bound F_1F_0 (0.2–0.5 mg/ml) were reacted with NBD-Cl for 60 min in the dark, in T8 buffer (50 mM TrisSO₄ pH 8.0), containing 2.5 mM MgSO₄ at room temperature then 50 μ l aliquots were transferred to 1ml of ATP assay buffer to determine ATPase activity. For protection from NBD-Cl inhibition, F_1F_0 membranes were preincubated 60 min with ADP or Pi at room temperature before addition of NBD-Cl. MgSO₄ and ADP or Pi were present in equimolar concentrations in the reaction assay. Blank controls contained protein in T8 buffer with no added NBD-Cl. Up to 10 mM of MgADP or 50 mM of MgPi alone were shown to have no inhibitory effect. [9, 15].

Reversal of NBD-Cl inhibited ATPase activity by DTT

For DTT induced reversal of NBD-Cl inhibition, membrane bound F_1F_o were first reacted with NBD-Cl (150 μ M) for 1 h at room temperature in the dark. Then, 4 mM DTT was added and incubation continued for 1 h at room temperature before ATPase assay. Control samples without NBD-Cl or DTT were included and incubated for the same time periods.

Inhibition of ATPase activity by azide, fluoroaluminate, or fluoroscandium

Azide induced inhibition was measured by preincubating membrane bound F_1F_o with varied concentrations of sodium azide for 30 min. Then, 1 ml ATPase assay buffer was added to measure the activity. Fluoroaluminate or fluoroscandium induced inhibition was measured by incubating membrane bound F_1F_o 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 0.2–0.5 mg/ml in the presence of varied concentrations of AlCl_3 or ScCl_3 . A 1 ml ATPase assay buffer was then added to 50 μ l reaction samples to measure the ATPase activity. Omission of MgSO_4 , NaADP, or NaF resulted in no inhibition of membranes.

Results

Growth properties of single and double mutants of *E. coli* ATP synthase

Nine new single mutants, α I346A, α I346D, α I346Q, α I346R, α I348A, α I348D, α I348Q, α I348R, and α R376A, and six double mutants, α I346R/ β R182A, α I346R/ β R246A, α I346R/ α R376A, α I348R/ β R182A, α I348R/ β R246A, and α I348R/ α R376A, were generated. Despite non-polar hydrophobic side chains, α Ile-346 and α Ile-348 are strongly conserved in the α VISIT-DG sequence and are also in close proximity to positively charged P_i binding residues in the catalytic sites of ATP synthase. α I346A and α I348A mutants were constructed to appreciate the role of non-polar hydrophobic side chains in P_i binding and the transition state. α I346Q and α I348Q mutants were designed to investigate the impact of the larger side chain of Gln on α I346 and α I348. α I346D, α I348D, α I346R, and α I348R were generated to determine the impact of negative and positive charges on the nearby known P_i binding residues β R246, β R182, and α R376. Growth properties of β R246A, β R182A, and α R376A were in excellent agreement with previously published data [3, 9, 12, 15, 31]. Our motivation for the double mutants α I346R/ β R182A, α I346R/ β R246A, α I346R/ α R376A, α I348R/ β R182A, α I348R/ β R246A, and α I348R/ α R376A was to determine if the presence of the Arg side chain on α I346R or α I348R could compensate for absence of the Arg side chain on β R182A or α R376A.

Table 2 shows that the introduction of Ala, Asp, Gln, or Arg as α I346A, α I346D, α I346Q, α I346R, α I348A, α I348D, α I348Q, and α I348R caused some loss of oxidative phosphorylation as represented by loss of growth on succinate and limiting glucose mediums. Replacement of Arg into Ala in three known P_i binding residues β R182A, β R246A, and α R376A resulted in significant loss of oxidative phosphorylation and reduction in ATPase activity. Considerable oxidative phosphorylation was retained by the double mutants α I346R/ β R182A, α I346R/ α R376A, and α I348R/ β R182A. Comparative specific ATPase activity values of membrane bound F_1F_o preparations containing wild-type, null, and mutant enzymes are also shown in Table 2. Variable reduction in ATPase activity

was observed for all mutant enzymes. Double mutants α I346R/ β R182A, α I346R/ α R376A, and α I348R/ β R182A augmented the ATPase activity by about 70-fold from 0.07, 0.065 to 4.61, 4.97, and 4.87, respectively. Purity and integrity of α and β subunits in wild-type and mutant membrane bound F_1F_0 were in excellent agreement with previously published data [8, 30]. This result also confirmed that the reduced ATPase in mutants was not due to contaminants, impaired assembly of ATP synthase, or loss of F_1 during membrane preparation, thus attributing the low mutant activities to mutant F_1 .

Inhibition of membrane bound F_1F_0 ATPase activity by NBD-Cl and reversal by DTT

In previous studies, both membrane bound F_1F_0 and purified F_1 preparations provided equivalent assay results [5, 8, 9, 14, 15, 30, 32–35]. Therefore, all the inhibition assays in this study were carried out using membrane bound F_1F_0 because it is convenient and less time consuming. Figure 3 shows NBD-Cl induced inhibition of wild-type and mutant membranes in the presence of varied concentrations of NBD-Cl. NBD-Cl resulted in complete inhibition of wild-type with no residual activity, which was consistent with previous studies [5, 8–12, 14, 15]. α I346A, α I346Q, α I346R, α I348A, α I348Q, α I346R/ β R182A, α I346R/ α R376A, and α I348R/ β R182A mutants were also almost completely inhibited. Variable degrees of inhibition were observed for other single or double mutants at about 59% for α I346D, 34% for α I348D, 40% for α I348R, 61% for α R376A, 54% for α I346R/ β R246A, 64% for α I348R/ β R246A, and 88% for α I348R/ α R376A (Fig. 3). Several examples of incomplete or partial inhibition of mutant enzymes by NBD-Cl are documented in previous studies [5, 8–12, 14, 15]. To validate that the maximal reaction with NBD-Cl had been reached, partially inhibited membrane bound F_1F_0 mutant enzymes were incubated with 150 μ M NBD-Cl for 1 h as in Figure 3, followed by an additional amount of 200 μ M NBD-Cl (totaling 350 μ M) and continuing the incubation for an extra hour before measuring ATPase activity. No significant change in inhibition confirmed that the reaction of NBD-Cl was complete and that fully reacted α I346D, α I348D, α I348R, α R376A, α I346R/ β R182A, α I348R/ β R246, and α I348R/ α R376A membrane bound F_1F_0 mutants retained residual activity (Fig. 4A). We also determined if inhibition by NBD-Cl could be reversed by the addition of the reducing agent DTT since reversibility by DTT has been shown to be indicative of reaction specificity at β Tyr-297 [36, 37]. For this experiment, DTT restored near normal ATPase activity in all cases (Fig. 4B), verifying that NBD-Cl reacts specifically with residue β Tyr-297 in the wild-type and in mutants.

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

Figure 5 shows the MgADP protection data against NBD-Cl in wild-type and mutant membrane bound F_1F_0 enzymes. Previous research has shown that protection of NBD-Cl-induced inhibition of wild-type and a variety of mutant membrane bound F_1F_0 or water soluble F_1 occurs at high concentrations of MgADP. In this study too, high concentrations of MgADP were required to protect wild-type and mutant enzymes against NBD-Cl inhibition. High MgADP concentrations efficiently keep the β E site occupied in time average and thus hold back the access to NBD-Cl by sterically obstructing the site [5, 8–15]. Therefore, these results prove that NBD-Cl is reacting in β E in the mutants and that the ATPase activities measured in the mutants are actually of ATP synthase enzyme and not due to any

contaminant. This scheme is consistent with the X-ray crystallographic studies of Orris *et al* concluding NBD-Cl reacts specifically in the β E catalytic site [7].

MgPi protection against NBD-Cl reaction is shown in Figure 6. Pi protected fully against NBD-Cl inhibition of ATPase activity in wild-type and in α I346A, α I346D, α I346Q, α I346R, α I348A, α I348D, α I348Q, α I348R, α I346R/ β R182A, α I346R/ α R376A, and α I348R/ β R182A mutants. Pi did not protect against NBD-Cl inactivation in α I346R/ β R246A, α I348R/ β R246A, or α I348R/ α R376A mutants.

Inhibition of ATPase activity by fluoroaluminate, fluoroscandium, and azide

Effects of transition and ground state analogs were studied. Figures 7A and 7B show the inhibition of wild-type and mutant enzymes by fluoroaluminate and fluoroscandium, respectively. AlCl_3 induced complete inhibition of wild-type, α I346A, α I346Q, and α I348A. Maximal inhibition by AlCl_3 for other mutants was observed at about 17% for α I346D, 84% for α I346R, 23% for α I348D, 86% for α I348Q, 0% for α I348R, 93% for α I346R/ β R182A, 32% for α I346R/ β R246A, 80% for α I346R/ α R376A, 84% for α I348R/ β R182A, 20% for α I348R/ β R246A, and 42% for α I348R/ α R376A. ScCl_3 induced nearly complete inhibition of wild-type. ScCl_3 caused variable degrees of inhibition at about 66% for α I346A, 0% for α I346D, 80% for α I346Q, 15% for α I346R, 84% for α I348A, 30% for α I348D, 65% for α I348Q, 30% for α I348R, 80% for α I346R/ β R182A, 6% for α I346R/ β R246A, 45% for α I346R/ α R376A, 75% for α I348R/ β R182A, 20% for α I348R/ β R246A, and 30% for α I348R/ α R376A in mutants. Sodium azide is a known potent inhibitor of ATP synthase. As seen in Figure 8, azide strongly inhibited wild-type enzyme and caused varied degrees of inhibition in mutants at about 44% for α I346A, 20% for α I346D, 20% for α I346Q, 43% for α I346R, 29% for α I348A, 0% for α I348D, 0% for α I348Q, 0% for α I348R, 69% for α I346R/ β R182A, 12% for α I346R/ β R246A, 20% for α I346R/ α R376A, 60% for α I348R/ β R182A, 0% for α I348R/ β R246A, and 0% for α I348R/ α R376A.

Discussion

The purpose of this study was to understand the functional importance of non-polar hydrophobic side chains of α Ile-346 and α Ile-348 residues in the highly conserved α VISIT-DG sequence of *E. coli* ATP synthase. All the VISIT-DG sequence residues are located in close proximity to the positively charged Pi binding residues in the catalytic sites. α I346 is only 2.98 and 3.63 Å from α R376 and β R182, two known Pi binding residues. β R246, another known Pi binding residue, is 7.41 Å apart from α I346. The distance between α I348 and β R182 is only 3.66 Å, but α R376 and β R246 are located at 5.29 and 5.77 Å (Fig. 1B). The side chains of α I346 and α I348 are also in close proximity to the bound Pi analogs AlF_3 , AlF_4^- , and SO_4^{2-} [16, 17]. AlF_4^- is located within 4.08 and 4.74 Å from α I346 and α I348 (Fig. 1). Pi binding is a prerequisite for ATP synthesis by ATP synthase. Mutagenic analysis along with molecular modulations provides the opportunity to elucidate and understand the functional role of residues in Pi binding.

In previously published studies that used inhibitory effects of Pi analogs and the MgPi protection assay against NBD-Cl inhibition on mutant and wild-type ATP synthase, we demonstrated the functional role(s) of Pi binding subdomain and surrounding residues in Pi

binding [5, 8–15]. Biochemical and biophysical characterization of nine catalytic site residues identified six residues, α Arg-376, β Arg-182, β Arg-246, β Lys-155, α Ser-347, and α Thr-349, directly or indirectly involved in Pi binding. While four residues, α Arg-376, β Arg-182, β Arg-246, and β Lys-155, were directly involved in Pi binding, the two residues, α Ser-347 and α Thr-349, supported Pi binding and transition state stabilization through their interaction with β Arg-246 and β Arg-182 [5, 8–15]. These residues were shown to form a triangular subdomain around the Pi analogs AlF_3 , AlF_4^- , or SO_4^{2-} within the catalytic site (Fig. 1B). Three other residues, β N243, α F291, and α G351, although important for function were not involved in direct Pi binding. In previous studies, we also demonstrated that the introduction of a negative or positive charge in Pi binding subdomain resulted in strong variations of Pi binding [8, 10, 14, 15], indicating the presence of charged residues in the vicinity of the triangle was an important determinant of Pi binding. In this study, we used a similar approach by replacing α Ile-346 and α Ile-348 residues with a variety of residues Ala, Asp, Gln, or Arg.

Construction of α I346A, α I346D, α I346Q, α I346R, α I348A, α I348D, α I348Q, α I348R, α I346R/ β R182A, α I346R/ β R246A, α I346R/ α R376A, α I348R/ β R182A, α I348R/ β R246A, or α I348R/ α R376A mutants did not affect assembly or structural integrity of the membrane bound ATP synthase. The content of F_1 - α and F_1 - β subunits in mutant enzymes was comparable to wild-type. As shown in Table 2, Mutations caused insignificant to substantial loss of oxidative phosphorylation and reduction in ATPase activity in mutant enzymes as suggested by growth on succinate or limiting glucose medium. A wide range of reduction in ATPase activity between 4-fold to 700-fold was observed for the mutant enzymes.

As shown in Figure 6, MgPi protected against NBD-Cl inhibition of all α Ile-346 and α Ile-348 single mutants, demonstrating Pi binding in the absence of α I346 or α I348. These results suggest that α -subunit VISIT-DG sequence residues α Ile-346 and α Ile-348 play no direct role in catalysis. While no Pi binding was observed in double mutants α I346R/ β R246A, α I348R/ β R246A, or α I348R/ α R376A, the double mutants α I346R/ β R182A, α I346R/ α R376A, and α I348R/ β R182A allowed Pi binding with substantial retention of oxidative phosphorylation and ATPase activity (Fig. 6). Clearly, insertion of Arg at α Ile-346 or α Ile-348 positions can compensate for the loss of Arg at β Arg-182 or α Arg-376 but not at β Arg-246.

MgADP-fluoroaluminate and MgADP-fluoroscandium are known potent inhibitors of wild-type *E. coli* ATP synthase, and both are believed to mimic the chemical transition state [5, 8–12, 14, 15, 31, 38]. Transition state-like structures involving bound MgADP- AlF_4^- complex have been seen in catalytic sites in ATP synthase by X-ray crystallography [17]. As shown in Figure 7, fluoroaluminate caused complete inhibition of wild-type, α I346A, α I346Q, and α I348A but resulted in variable degrees of inhibition for other mutants, indicating a partial to strong destabilization of the transition state. As expected, fluoroscandium also induced almost complete inhibition of wild-type enzyme but caused nearly complete to no inhibition of mutants, suggesting no destabilization to strong destabilization of the transition state. These results correspond with the amount of oxidative phosphorylation and ATPase activity found in each of the mutants. The higher degree of inhibition in double mutants α I346R/ β R182A, α I346R/ α R376A, and α I348R/ β R182A

indicates some rescue of the transition state. Overall, these results suggest α Ile-346 and α Ile-348 play a direct role in the transition state.

Azide induced X-ray crystallographic studies of ATP synthase [39] showed that azide inhibits ATP synthase by forming a tight-binding MgADP-azide complex in β DP catalytic sites resembling MgADP-beryllium fluoride. Therefore, azide may be considered an analog of the MgATP ground-state. In the MgADP-azide complex, the azide position corresponds to MgATP gamma phosphate [39]. As shown in Figure 8, azide caused almost complete inhibition of wild-type membrane bound F_1F_0 and resulted in variable degrees of inhibition for mutants. Thus, all mutants seem to have some effect on substrate binding because of an effect at the gamma-P position. In single molecule experiments, Pi binding and release events have been directly linked to rotation of the central stalk [40]. Perturbation of the Pi binding site might disturb the integrity of the link between Pi binding and rotation and result in uncoupling. Therefore, α Ile-346 and α Ile-348 mutation data strongly suggest that both residues are required for transition state stabilization and not for Pi binding.

Because Arg residues occur frequently in catalytic sites in proteins (30), modulating the number of Arg residues in the Pi binding subdomain of ATP synthase is an advantageous approach to study their role in catalysis or Pi binding. Residues α Ile-346 and α I348 are 3.63Å and 3.66 Å, 7.41Å and 5.77 Å, 2.98Å and 5.29 Å from known Pi binding residues β Arg-182, β R246, and α Arg-376, respectively. Phosphate analogs AlF_4 and SO_4^{2-} are 4.08 Å and 5.65 Å from α I346 and 4.74 Å and 6.56 Å from α I348. The distance between α I346 and α I348 is 5.10 Å (nearest atom distances quoted in all cases) using PDB 1H8E coordinates [17]. Thus, one of the experimental approaches we used was to introduce the mutation α I346R or α I348R in the wild-type background (with β Arg-182, β Arg-246, α Arg-376) and in the presence of the β R182A, β R246A, or α R376A mutation. The position of α I346 and α I348 on top of the Pi binding pocket across the catalytic α/β interface with side-chains pointing toward the bound Pi analogs also makes α I346 and α I348 suitable candidates for the insertion of a new Arg. Introduction of Arg at α I346R or α I348R places extra positive charge fairly close to Pi. Further, double mutations α I346R/ β R182A, α I346R/ β R246A, α I346R/ α R376A, α I348R/ β R182A, α I348R/ β R246A, and α I348R/ α R376A will allow the Arg to fit into the large “hole” generated by β Ala-182, β Ala-246, or α Ala-376 mutation. The β R182A, β R246A, or α R376A mutants did not allow Pi binding, but the α I346R or α I348R “rescued” Pi binding in combination with β R182A or α R376A but not with β R246A (Fig. 6). Previously [9] it was observed that introduction of Lys as a positive in place of Arg in the form of β R246K could not compensate for the loss of Arg from the known Pi binding residue β R246 therefore we have focused on introduction of Arg residue. Moreover, Lys in many other studies failed to compensate for the loss of Arg (Z. Ahmad unpublished data). Although, not checked in this study but it would be interesting to see if Lys can support the loss of Arg in phosphate binding subdomain.

Based on the loss of oxidative phosphorylation as manifested by growth on succinate or limiting glucose medium along with an almost 6-fold lower ATPase activity, α Arg-346 and α Arg-348 could be expected to assume the identical stereochemical interactions of β Arg-182 or α Arg-376. Electrostatic interactions are critical for the catalysis process, and we conclude that the presence of at least one positive charge at this general location is an

essential determinant for the initiation of Pi binding in β E catalytic site. In addition, extra positive charge in the form of α I346R or α I348R or extra negative charge in the form of α I346D or α I348D in wild-type background did not abrogate Pi binding (Fig. 6). Although, we previously found that the presence of negative charge in the form of α T349D prevented Pi binding by virtue of neutralizing the positive charge of nearby residue β Arg-182 [15].

In summary, the highly conserved α -subunit VISIT-DG sequence residues α Ile-346 and α Ile-348 in *E. coli* ATP synthase seem to be required for function and transition state stabilization but appear to have no direct role in Pi binding. Introduction of Arg at these sites can compensate for the absence of Arg at known Pi binding residue sites β Arg-182 or α Arg-376.

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Abbreviations used

NBD-Cl	7-chloro-4-nitrobenzo-2-oxa-1,3-diazole
DTT	dithiothreitol
EDTA	Ethylenediaminetetraacetic acid
TES	2-[Tris(hydroxymethyl)methylamino]-1-ethanesulfonic acid
DMSO	dimethyl sulfoxide

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Highlights

- Role of highly conserved α I346 and α I348 VISIT-DG sequence residues in Pi binding is proposed
- Both α I346 and α I348 residues are required for upholding the phosphate binding sub-domain.
- Both α I346 and α I348 residues are required for the transition state stabilization.
- Introduction of Arg in place of Ile can compensate for the loss of an Arg involved in Pi binding

Figure 1. X-ray structures of ATP synthase catalytic sites showing spatial relationship of α -VISIT-DG sequence residue α I346 and α I348

(A) The β ADP+Pi (β -half-closed) site in the AlF_4^- -inhibited enzyme [17]. The sulfate ion is thought to occupy the position of natural Pi. (B) The β DP site in the AlF_4^- -inhibited enzyme. [17]. Figure was generated by PDB files 1H8E using Rasmol software. Residue numbers are based on *E. coli* numbering.

Figure 2. Immunoblot of wild-type purified F₁, wild-type membrane bound F₁F_o, and mutant membrane bound F₁F_o ATP synthase with anti-F₁-α antibody

Wild-type (WT) purified F₁ (0.4μg), WT membrane bound F₁F_o, and mutant membrane bound F₁F_o preparations (4μg) were run on 10% SDS-polyacrylamide gel along with null mutant pUC118 (4μg). Protein bands were transferred to nitrocellulose and immunoblotted using anti-F₁-α antibody.

Figure 3. Inhibition of wild-type and mutant membrane bound F_1F_0 ATP synthase by NBD-Cl Membranes were preincubated for 60 min at room temperature with varied concentrations of NBD-Cl. 1 ml ATP cocktail assay buffer was added to start the reaction. More details are provided in the Materials and Methods. A: wild-type (○); B: α I346A (○), α I346D (□), α I346Q (△), and α I346R (◇); C: α I348A (○), α I348D (□), α I348Q (△), and α I348R (◇); D: α R376A (○); E: α I346R/ β R182A (○), α I346R/ β R246A (□), and α I346R/ α R376A (△); F: α I348R/ β R182A (○), α I348R/ β R246A (□), and α I348R/ α R376A (△). Each data point represents an average of at least three experiments with duplicate samples, using 2–3 independent membrane bound F_1F_0 preparations. Results agreed within $\pm 10\%$.

Figure 4. Effect of an extra pulse of NBD-Cl and reversal of NBD-Cl effect in the presence of DTT

A: Membrane bound F_1F_0 ATP synthase was inhibited with 150 μ M NBD-Cl for 60 min under conditions as described in Fig. 3. Then, an additional amount of 200 μ M NBD-Cl was added and incubation continued for 1 hr before assay. B: Membrane bound F_1F_0 ATP synthase (Mbr) was incubated with or without 150 μ M NBD-Cl for 60 min under conditions as described in Fig. 3. The degree of inhibition was examined. In parallel samples, 4 mM DTT was then added, and incubation continued for a further 60 min before assay. Each bar graph represents wild-type, α I346D, α I348D, α I348R, α R376A, α I346R/ β R246A, and α I348R/ β R246A from left to right. For clarity, only mutants retaining residual activity after NBD-Cl inhibition are shown.

Figure 5. MgADP induced protection against NBD-Cl inhibition

Wild-type and mutant membrane bound F_1F_0 were preincubated for 1h at room temperature with varied concentrations of MgADP, then 150 μ M NBD-Cl was added, and incubation continued at room temperature in the dark for 1 hour. Aliquots were assayed for ATPase activity. A: wild-type (○), α I346A (◐), α I346D (◻), α I346Q (◑), and α I346R (◈); B: wild-type (○), α I348A (◐), α I348D (◻), α I348Q (◑), and α I348R (◈); C: wild-type (○), α I346R/ β R182A (◐), α I346R/ β R246A (◻), and α I346R/ α R376A (◑); D: wild-type (○), α I348R/ β R182A (◐), α I348R/ β R246A (◻), and α I348R/ α R376A (◑). Results are means of at least three independent experiments and agreed within $\pm 10\%$.

A

B

C

Figure 6. MgPi protection against NBD-Cl inhibition of membrane bound F_1F_0 ATPase activity
 F_1F_0 membranes were preincubated with MgPi at zero, 2.5, 5, or 10 mM concentration as shown, for 60 min at room temperature. Then 150 μ M NBD-Cl was added and aliquots withdrawn for assay at time intervals as shown. Remaining ATPase activity was plotted against time of incubation with NBD-Cl. ○, no Pi added; □, 2.5 mM Pi; △, 5 mM Pi; ◇, 10 mM Pi. Each data point represents the average of at least three individual experiments using 2–3 independent membrane preparations. Results agreed within $\pm 10\%$.

A

Figure 7. Fluoroaluminumate and fluoroscandium induced inhibition of membrane bound F_1F_0 ATPase activity

Membranes were preincubated for 60 min at room temperature with 1 mM MgADP, 10 mM NaF, and reported concentrations of $AlCl_3$ (7A) or $ScCl_3$ (7B). Then 1 ml of ATP cocktail was added and ATPase activity determined. A: wild-type (○), $\alpha I346A$ (◊), $\alpha I346D$ (◻), $\alpha I346Q$ (◐), and $\alpha I346R$ (◑); B: wild-type (○), $\alpha I348A$ (◊), $\alpha I348D$ (◻), $\alpha I348Q$ (◐), and $\alpha I348R$ (◑); C: wild-type (○), $\alpha I346R/\beta R182A$ (◊), $\alpha I346R/\beta R246A$ (◻), and $\alpha I346R/\alpha R376A$ (◐); D: wild-type (○), $\alpha I348R/\beta R182A$ (◊), $\alpha I348R/\beta R246A$ (◻), and $\alpha I348R/\alpha R376A$ (◐). All the data points are means of at least three duplicate experiments using 2–3 independent membrane preparations. Variation was $\pm 10\%$ between different experiments.

Figure 8. Sodium azide induced inhibition of membrane bound F_1F_0 ATPase activity

Sodium azide was added directly to the membranes and incubated for 30 min before ATPase assay. More details are provided in materials and Methods. A: wild-type (○), $\alpha I346A$ (◐), $\alpha I346D$ (◑), $\alpha I346Q$ (◒), and $\alpha I346R$ (◈); B: wild-type (○), $\alpha I348A$ (◐), $\alpha I348D$ (◑), $\alpha I348Q$ (◒), and $\alpha I348R$ (◈); C: wild-type (○), $\alpha I346R/\beta R182A$ (◐), $\alpha I346R/\beta R246A$ (◑), and $\alpha I346R/\alpha R376A$ (◒); D: wild-type (○), $\alpha I348R/\beta R182A$ (◐), $\alpha I348R/\beta R246A$ (◑), and $\alpha I348R/\alpha R376A$ (◒). All the data points are means of at least three different

experiments using 2–3 independent membrane preparations. Variation was $\pm 10\%$ between different experiments.

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TABLE 1

List of oligonucleotides

Plasmids	Mutations	Sequence
pZA65	α I346A	CGACCAACGTG GCGT CCATTACCGATGG
pZA66	α I346D	CCGACCAACGTAG ACT CCATTACCGATGG
pZA67	α I346Q	CCGACCAACGTAC AGT CCATTACCGATGG
pZA68	α I346R	CGACCAACGTA AGATCT ATTACCGATGG
pZA69	α I348A	CCAACGTAATCAG CGCT ACCGATGGTCAG
pZA70	α I348D	CCAACGTAATCTCAG AT ACCGATGGTCAG
pZA71	α I348Q	CCAACGTAATCTCC AG ACCGATGGTCAG
pZA72	α I348R	CGTAATCTCT AGA ACCGATGGTCAGATC
pZA24	β R182A ^a	GGCGTAGGTGA AGCT ACTCGTGAGGG
pZA73	α R376A	CGGGTATTTCCGTATC GGCCG TTGGTGGTGCAGC
pZA7	β R246A ^b	GACAACATCTAT GCA TACACCCTGGC
pZA74	α I346R/ α R182A	pZA24 fragment added on plasmid pZA68
pZA75	α I346R/ α R376A	pZA73 fragment added on plasmid pZA68
pZA76	α I346R/ β R246A	pZA768 fragment added on plasmid pZA7
pZA77	α I348R/ α R182A	pZA24 fragment added on plasmid pZA72
pZA78	α I348R/ α R376A	pZA73 fragment added on plasmid pZA72
pZA79	α I348R/ β R246A	pZA72 fragment added on plasmid pZA7

Bold and underline bases introduce the mutation and new restriction site.

^a taken from ref (9)

^b taken from ref (15)

TABLE 2

Effects of single and double α I346 and α I348 mutations on cell growth and ATPase activity

^a Mutation	^b Growth On succinate	Growth yield in in limiting glucose (%)	^c ATPase Activity ($\mu\text{mol}/\text{min}/\text{mg}$)
Wild-type	++++	100	28
Null	–	47	0
α I346A	+++	90	6.9
α I346D	++	76	2.56
α I346Q	++	67	6.81
α I346R	++	74	3.74
α I348A	+++	93	5.54
α I348D	++	78	0.08
α I348Q	++	66	4.54
α I348R	+	64	0.04
α R182A	–	47	0.07
α R376A	–	56	0.065
β R246A ^d	–	50	0.25
α I346R/ α R182A	+++	77	4.61
α I346R/ α R376A	+++	83	4.97
α I346R/ β R246A	+	60	1.24
α I348R/ α R182A	+++	79	4.87
α I348R/ α R376A	+	53	1.32
α I348R/ β R246A	+	55	0.95

^aWild-type, pBWU13.4/DK8; Null, pUC118/DK8. All mutants were expressed with the β Y331W mutation also present, which does not significantly affect growth. Data are means of four experiments each.

^bGrowth on succinate plates after 3 days estimated by eye. +++, heavy growth; ++, substantial growth; +, light growth; –, very light growth; –, no growth. Qualitative growth estimation on succinate plates is a widely used and more reliable method than the growth estimation on liquid succinate media as the rate of growth for wild-type and mutants could be different.

^cATPase activity measured at 37°C and expressed as μmol ATP hydrolyzed/min/mg of membrane protein. Individual experimental points are the mean of duplicate assay tubes. Data are derived from at least two separate membrane preparations. Results from separate membrane preparations were in excellent agreement within $\pm 10\%$.

^dData taken from ref (9)