Significance of αThr-349 in the Catalytic Sites of *Escherichia coli* ATP Synthase

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**ABSTRACT:** This paper describes the role of α-subunit VISIT-DG sequence residue αThr-349 in the catalytic sites of *Escherichia coli* F₁F₀ ATP synthase. X-ray structures show the highly conserved αThr-349 in the proximity (2.68 Å) of the conserved phosphate binding residue βR182 in the phosphate binding subdomain. αT349A, -D, -Q, and -R mutations caused 90–100-fold losses of oxidative phosphorylation and reduced ATPase activity of F₁F₀ in membranes. Double mutation αT349R/βR182A was able to partially compensate for the absence of known phosphate binding residue βR182. Azide, fluorothiolate, and fluoroscandium caused insignificant inhibition of αT349A, -D, and -Q mutants, slight inhibition of the αT349R mutant, partial inhibition of the αT349R/βR182A double mutant, and complete inhibition of the wild type. Whereas NBD-Cl (7-chloro-4-nitrobenzo-2-oxa-1,3-diazole) inhibited wild-type ATPase and its αT349A, -D, -R, and -Q mutants essentially completely, βR182A ATPase and double mutant αT349A/βR182A were inhibited partially. 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 the wild type, αT349R, and double mutant αT349R/βR182A but not in αT349A, αT349D, or αT349Q. The results demonstrate that αThr-349 is a supplementary residue involved in phosphate binding and transition state stabilization in ATP synthase catalytic sites through its interaction with βR182.

In a 75 year life span, a typical 70 kg human generates approximately 2.0 million kg of ATP. The cell’s energy currency is generated by converting food into useable energy by oxidation. F₁F₀ ATP synthase is responsible for the fundamental means of cell energy production in animals, plants, and almost all microorganisms, which occurs by oxidation or photophosphorylation in membranes of bacteria, mitochondria, and chloroplasts. ATP synthase is one of the smallest biological nanomotors and is structurally similar in all species.1–4 In its simplest form, as in *Escherichia coli*, it contains eight different subunits distributed in the water-soluble F₁ sector (subunits α₁β₂γδε) and the membrane-associated F₀ sector (subunits abcf₁₀). The total molecular size is ~530 kDa.5 In chloroplasts, there are two isoforms of subunit b. In mitochondria, there are seven to nine additional subunits, depending on the source, but in total, they contribute only a small fraction of additional mass and may have regulatory roles.5–7

The membrane-bound F₁F₀ ATP synthase enzyme is highly conserved and structurally identical among different species. X-ray structures of bovine enzyme8 established the presence of three catalytic sites at α-subunit/β-subunit interfaces of the αβγ hexamer. ATP hydrolysis and synthesis occur in the F₁ sector, whereas proton transport occurs through the membrane-embedded F₀.8,9 ATP synthase is a result of proton gradient-driven clockwise rotation of γ (as viewed from the outer membrane), while ATP hydrolysis results in anticlockwise rotation of the γ-subunit. Detailed reviews of ATP synthase structure and function may be found in refs 10–18.

A precise knowledge of Pᵢ (inorganic phosphate) binding is not only essential for following the reaction mechanism of ATP synthesis and hydrolysis but also equally important for understanding the relationship between catalytic mechanism and mechanical rotation in this biological nanomotor. For this reason, we have focused our efforts on determining the role of conserved residues in and around catalytic site Pᵢ binding subdomain.9 Knowledge of Pᵢ binding residues and residues surrounding the Pᵢ binding subdomain is imperative for (i) the molecular modulation of the catalytic site(s) for the improved catalytic and motor function of this enzyme, (ii) an explanation of how ATP synthase binds ADP and Pᵢ within its catalytic sites in the face of a relatively high ATP/ADP concentration ratio, and (iii) understanding the relationship between Pᵢ binding and subunit rotation.20–22 Many earlier attempts to measure Pᵢ binding in purified *E. coli* F₁ failed to detect appreciable Pᵢ binding at physiological Pᵢ concentrations.21,23,24 but modification of the assay devised by Perez et al.25 provides a useful measure of Pᵢ binding. In this assay, protection is afforded by Pᵢ against inhibition of ATPase activity induced by covalent reaction with 7-chloro-4-nitrobenzo-2-oxa-1,3-diazole (NBD-
X-ray crystallography showed that the covalent interaction of NBD-Cl specifically with β297 occurs in the βE catalytic site (Figure 1A); thus, protection afforded by P_i indicates that binding of P_i occurs at the βE catalytic site. Modification of the assay described above for E. coli, purified F_1 or F_1F_0 membranes, previously allowed us to investigate the relationship between P_i binding and catalysis for eight residues, namely, βArg-246, βAsn-243, αArg-376, βLys-155, βArg-182, αPhe-291, αSer-347, and αGly-351. Although all these residues are situated in the proximity of the phosphate analogues AlF_4^- or SO_4^{2-} in X-ray structures of catalytic sites, we found that four residues, namely, βArg-246, αArg-376, βLys-155, and βArg-182, grouped in a triangular fashion, are directly involved in P_i binding while the fifth residue, αSer-347, is indirectly involved in P_i binding through its interaction with βArg-246 (see Figure 1B).26,27,28

The mechanism of condensation of P_i with MgADP proposed by Senior et al.3 was strengthened by the X-ray crystallography structure of bovine ATP synthase of Menz et al.28 showing the transition state analogue MgADP trapped in catalytic sites (Figure 1B). It is clear from the geometry of this complex that the fluoroumalinate group occupies the position of the ATP γ-phosphate in the predicted transition state. The first transition state-like structure of F_1 from rat liver crystallized with the P_i analogue vanadate (V_50), reported by Pedersen’s group, demonstrated that ADP was not essential, suggesting that the MgV_50F_1 complex inhibited the catalytic activity to the same extent as that observed for the MgADP−γP_iF_1 complex.36 Neither purified F_1 nor membrane-bound F_1F_0 from E. coli is inhibited by MgV_50 or MgADP−γP_i. Consequently, we have relied on inhibition of ATPase activity by fluoroumalinate (or fluoroscandium) to assess the potential to stabilize a transition state complex.26,27,28,32,35

Cingolani and Duncan18 have resolved the first E. coli F_1 sector high-resolution crystal structure in an autoinhibited conformation. This structure divulges a wealth of information about the regulatory features of bacterial and chloroplast ATP synthase. Moreover, the E. coli ATP synthase X-ray structure paves the way for the development of new antimicrobial drugs.

For E. coli, ATP synthase is naturally a better candidate for antimicrobial drugs in comparison to mitochondrial ATP synthase.4,13 New drug discovery to combat tuberculosis disease targeting bacterial ATP synthase corroborates this assertion.37 Because the E. coli high-resolution structure does not contain sulfate, phosphate, fluoroumalinate, or fluoroscandium, we have relied on the mitochondrial ATP synthase structure that is very similar to that of E. coli (∼70% homologous sequence) as this study deals with the analogues described above.7,32,38

Fortunately, by mutagenic analysis along with the NBD-Cl protection assay, as well as ATPase inhibition by transition state analogues, we can investigate the direct or indirect role of residues in P_i binding. In this work, we examine the role of the highly conserved α-subunit VISIT-DG sequence residue Thr-349 in the process of P_i binding. Figure 1B shows the position of αThr-349 with respect to other known P_i binding residues. The strategic position of αThr-349 in the P_i binding subdomain leads to the following basic questions: Is αThr-349 involved in P_i binding directly or indirectly? Do the αThr349A, αThr349D, αThr349Q, and αThr349R mutations have any effect on transition state formation? Also, can αThr349R compensate for βArg-182, a known P_i binding residue?

### MATERIALS AND METHODS

#### Construction of Wild-Type and Mutant Strains of E. coli

The strain for wild-type E. coli was pBWU13.4/DK8.39 All the mutants were generated by the method of Vandeyar et al.40 The M13mp18 template containing the HindIII−XbaI fragment from pSN6 was used for oligonucleotide-directed mutagenesis. Plasmid pSN6 contains the βY331W mutation from plasmid pSWM42 introduced on a ScaI−EagI fragment into pBWU13.4,30 which expresses all the ATP synthase genes. The following mutagenic oligonucleotides were used: αThr349A, GTAATCTCTATAGCCGATGGTCAGATC, where the underlined bases introduce the mutation and a new SacI restriction site; αThr349D, GTAATCTCATGGGATGGTCAGATC, where the underlined bases introduce the mutation and a new MfeI restriction site; αThr349Q, GTAATCTCCATTCAGGATGGTCAGATC, where the underlined base in-
Introduces new mutation αT349Q (ACC → CAG); αT349R, CGTAATCTCCTATTGGCTGATGGTCAGATC, where the underlined bases introduce the mutation and a new NruI restriction site; βR182A, GGGCTAGGTTAAGCTAATTGCTGAGGG, where the underlined bases introduce the mutation and a new AluI restriction site. DNA sequencing was performed to confirm the presence of mutations and the absence of undesired changes in sequence, and the mutations were transferred to pSN6 on a Csp451 (an isoschizomer of BstBI) and the PmlI fragment generating the new plasmids pZA20-(αT349A/βY331W), pZA21(αT349D/βY331W), pZA22-(αT349Q/βY331W), pZA23(αT349R/βY331W), and pZA24-(βR182A/βY331W). Double mutant pZA25 (αT349R/βR182A) was generated by combining the pZA23 fragment on the pZA24 plasmid at the Csp451 and PmlI site. Each plasmid was transformed into strain DK842 containing a plasmid at the Csp451 and PmlI site. Each plasmid was transformed into strain DK842 containing a plasmid at the Csp451 and PmlI site.

ATPase Activity of Membranes.

Growth Yield in Limiting Glucose Medium, and Assay of ATPase Activity of Membranes. E. coli membrane-bound F1Fo were prepared by the method of Senior et al. Notably in this procedure, F1Fo-bound membrane initial pellets are washed three times. The first wash is conducted in 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 performed in buffer containing 5 mM TES (pH 7.0), 15% glycerol, 40 mM 6-aminohexanoic acid, 5 mM p-aminobenzamidine, 0.5 mM dithiothreitol (DTT), and 0.5 mM EDTA. Before the experiments, membranes were washed twice more by resuspension and ultracentrifugation in 50 mM TrisSO4 (pH 8.0) and 2.5 mM MgSO4. These extra washes are included for possible future use.

Preparation of E. coli Membranes, Measurement of Growth Yield in Limiting Glucose Medium, and Assay of ATPase Activity of Membranes. E. coli membrane-bound F1Fo were prepared by the method of Senior et al. Notably in this procedure, F1Fo-bound membrane initial pellets are washed three times. The first wash is conducted in 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 performed in buffer containing 5 mM TES (pH 7.0), 15% glycerol, 40 mM 6-aminohexanoic acid, 5 mM p-aminobenzamidine, 0.5 mM dithiothreitol (DTT), and 0.5 mM EDTA. Before the experiments, membranes were washed twice more 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 truly zero activity. Therefore, the low activities with the mutants must be coming from the mutants, not from any other contaminants. The experiments are performed to make sure that growth yield in limiting glucose was measured as described previously.

Measurement of ATPase activity was performed in 1 mL of assay buffer containing 10 mM NaATP, 4 mM MgCl2, and 50 mM TrisSO4 (pH 8.5) at 37 °C. Reactions were started by the addition of membrane-bound F1Fo and stopped by addition of 1 mL of sodium dodecyl sulfate (SDS) to a final concentration of 3.3%. Release of Pi was measured as described in ref 45. Reaction times for the wild-type F1Fo, membrane (20–30 μg of protein) were 5–10 min, while reaction times for F1Fo-bound mutant membranes (40–60 μg of protein) were 30–50 min. All reactions were found to be linear with time and protein concentration. The purity and integrity of membranes were checked by SDS gel electrophoresis on 10% acrylamide gels as described in ref 46 and immunoblotting with rabbit polyclonal anti-F1α and anti-F1β antibodies as described in ref 47.

Inhibition of ATPase Activity by NBD-Cl and Protection by MgADP or Pi. A stock solution of NBD-Cl was prepared in dimethyl sulfoxide (DMSO) and protected from light. F1Fo-bound membranes (0.2–0.5 mg/mL) were reacted with NBD-Cl for 60 min in the dark, at room temperature, in T8 [50 mM TrisSO4 (pH 8.0)] and 2.5 mM MgSO4. ATPase activity was determined by adding 50 μL aliquots from the assay described above to 1 mL of ATPase assay buffer. For protection from NBD-Cl inhibition by ADP or Pi, membranes were preincubated for 60 min with a protecting agent at room temperature before the addition of NBD-Cl. MgSO4 and ADP or Pi were present at equimolar concentrations in the reaction assay. Control samples containing the ligand without added NBD-Cl were included. Neither MgADP (up to 10 mM) nor Pi (up to 50 mM) had any inhibitory effect alone.

Reversal of NBD-Cl-Inhibited ATPase Activity by DTT. To determine the DTT-induced reversal of NBD-Cl inhibition, F1Fo-bound membranes were first reacted with NBD-Cl (150 μM) for 1 h at room temperature in the dark, and then DTT (final concentration of 4 mM) was added and incubation continued for 1 h at room temperature before the ATPase assay. Control samples without NBD-Cl and/or DTT were incubated for the same amounts of time.

Inhibition of ATPase Activity by Azide, Fluoroaluminate, or Fluoroscadium. For measurement of azide inhibition, membrane-bound F1Fo was preincubated with varied concentrations of sodium azide for 30 min. Then 1 mL of ATPase assay buffer was added to measure the activity. Measurements of fluoroaluminate or fluoroscadium inhibition were performed by incubating membrane-bound F1Fo, for 60 min at room temperature in 50 mM TrisSO4, 2.5 mM MgSO4, 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 AlCl3 or ScCl3 (see Results); 50 μL aliquots were then added to 1 mL of ATPase assay buffer, and activity was measured as described above. It was confirmed in control experiments that no inhibition was seen if MgSO4, NaADP, or NaF was omitted.

Inhibition of ATPase Activity by Dicyclohexylcarbodiimide (DCCD). The method of Weber et al. was used to covalently modify the wild-type and mutant F1Fo membrane by DCCD. Measurement of ATPase activity was done by adding 1 mL of ATPase assay buffer containing 10 mM NaATP, 4 mM MgCl2, and 50 mM TrisSO4 (pH 8.5) at 37 °C to the 100 μL aliquots of 16 h DCCD-modified ATP synthase.

RESULTS

Growth Properties of αT349A, αT349D, αT349Q, αT349R, βR182A, and αT349R/βR182A Mutants of E. coli ATP Synthase. Five new single mutants, αT349A, αT349D, αT349Q, αT349R, and βR182A, and one double mutant, αT349R/βR182A, were generated. Residue αThr-349 was chosen for mutagenesis because of its high level of conservation in the α-subunit VISIT-DG sequence and proximity to the Pi binding pocket. The αT349A mutant was used to appreciate the role of the Thr-OH side chain in Pi binding and the transition state. The αT349Q mutant was designed to understand the impact of the larger side chain of Gln on αThr-349. αT349D and αT349R were constructed to establish the impact of negative and positive charge on the nearby βR182, a known Pi binding residue. It should be noted here that the growth properties of βR182A were in excellent agreement with those published previously for the purified F1 E. coli ATP synthase. The motivation behind double mutant αT349R/βR182A was to determine if Arg on αT349 could compensate for the absence of Arg on βR182A.

Table 1 shows that introduction of Ala, Asp, Gln, or Arg as αT349A, αT349D, αT349Q, αT349R, and βR182A resulted in a loss of oxidative phosphorylation. All mutations barred growth on succinate-containing medium, and growth yields in

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published data and yielded results that were in excellent agreement with previously published data and conformed to the site occupied by MgADP in time average and thus hold back the access to NBD-Cl by sterically obstructing the site.\textsuperscript{19,29} Earlier, we have shown that MgADP protects against NBD-Cl inhibition. Membranes were preincubated for 60 min at room temperature with varied concentrations of NBD-Cl, then aliquots were added to 1 mL of assay buffer, and ATPase activity was determined. Details are given in Materials and Methods. Symbols: (\textbullet) wild type, (○) \textit{α}T349A, (□) \textit{α}T349D, (◇) \textit{α}T349Q, (△) \textit{α}T349R, (▽) \textit{β}R182A, and (⋆) \textit{α}T349R/\textit{β}R182A. Each data point represents an average of at least four experiments, using two or three independent membrane preparations of each mutant. Results agreed within ±10%.

Table 1. Effects of \textit{α}T349A, -D, -Q, and \textit{α}T349R/\textit{β}R182A Mutations on Cell Growth and ATPase Activity

| species\textsuperscript{a} | growth on succinate\textsuperscript{b} | growth yield in limiting glucose (%) | ATPase activity\textsuperscript{c} (\textmu\text{mol} min\textsuperscript{-1} mg\textsuperscript{-1}) |
|----------------|-----------------|-----------------|-----------------|
| wild type | +++++ | 100 | 28 |
| null | − | 46 | 0 |
| \textit{β}Y331W | +++++ | 95 | 26 |
| \textit{α}T349A | ± | 51 | 0.30 |
| \textit{α}T349D | ± | 49 | 0.28 |
| \textit{α}T349Q | ± | 47 | 0.29 |
| \textit{α}T349R | ± | 50 | 0.31 |
| \textit{α}T349R/\textit{β}R182A | ++ | 66 | 4.40 |

\textsuperscript{a}Wild type, pBWU1134/DK8; null, pUC118/DK8. \textit{α}T349A, -D, -Q, and \textit{β}R182A mutants were expressed with the \textit{β}Y331W mutation also present, which does not significantly affect growth. Data are means of four to six experiments each. \textsuperscript{b}Growth on succinate plates after 3 days estimated by eye: ++++, heavy growth; +, substantial growth; ±, very light growth; −, no growth. \textsuperscript{c}ATPase activity measured at 37 °C and expressed as micromoles of ATP hydrolyzed per minute per milligram of 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 within ±10%.

Figure 2. Inhibition of membrane-bound wild-type and mutant ATP synthase by NBD-Cl. Membranes were preincubated for 60 min at room temperature with varied concentrations of NBD-Cl, then aliquots were added to 1 mL of assay buffer, and ATPase activity was determined. Details are given in Materials and Methods. Symbols: (\textbullet) wild type, (○) \textit{α}T349A, (□) \textit{α}T349D, (◇) \textit{α}T349Q, (△) \textit{α}T349R, (▽) \textit{β}R182A, and (⋆) \textit{α}T349R/\textit{β}R182A. Each data point represents an average of at least four experiments, using two or three independent membrane preparations of each mutant. Results agreed within ±10%.

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Inhibition of ATPase Activity of ATP Synthase in Membranes by NBD-Cl and Reversal by Dithiothreitol.

All the inhibition assays were conducted using membrane-bound \textit{F}_{1}\textit{F}_{0} for both membrane preparations and the purified \textit{F}_{1} preparation, provide equivalent assay results, and are highly convenient and less time-consuming.\textsuperscript{19,29,30,35,49−52} Figure 2 shows NBD-Cl-induced inhibition of wild-type and mutant membranes in the presence of varied concentrations of NBD-Cl. NBD-Cl caused potent inhibition of the wild type with no residual activity, and this is consistent with previous studies.\textsuperscript{19,29−33,35} The \textit{α}T349A mutant was also almost completely inhibited, while \textit{α}T349D, \textit{α}T349Q, \textit{α}T349R, \textit{β}R182A, and \textit{α}T349R/\textit{β}R182A were inhibited by ∼85, 90, 90, 60, and 50%, respectively, with ∼10−50% residual activity. In previous studies, we have noted several instances in which mutant or wild-type ATP synthase was incompletely inhibited by inhibitors like fluoroaluminate, fluorescandium, sodium azide, NBD-Cl, polyphenols, and peptides.\textsuperscript{19,29−33,35,49−52} To authenticate that maximal reaction with NBD-Cl had been achieved, we incubated each membrane-bound \textit{F}_{1}\textit{F}_{0} preparation with 150 \textmu M NBD-Cl for 1 h as in Figure 2, followed by a supplementary amount of 200 \textmu M NBD-Cl (totaling 350 \textmu M) and continuing the incubation for an extra hour before assaying ATPase activity. As expected, very little or no additional inhibition occurred (Figure 3A). This demonstrates that the reaction of NBD-Cl was complete and that fully reacted \textit{α}T349D, \textit{α}T349Q, \textit{α}T349R, \textit{β}R182A, and \textit{α}T349R/\textit{β}R182A mutant \textit{F}_{1}\textit{F}_{0} membranes retained residual activity. Subsequently, we checked if inactivation by NBD-Cl could be reversed by addition of the reducing agent DTT because reversibility by DTT was indicative of specificity of reaction in previous studies. In this case, wild-type and mutant enzymes were preincubated with 150 \textmu M NBD-Cl as in Figure 2 and then 4 mM DTT was added and incubation continued for 1 h before the ATPase assay. It can be seen in Figure 3B that DTT completely restored full activity in all cases. This proves that NBD-Cl reacts specifically with residue βTyr-297 in the wild type as well as in six other mutants.\textsuperscript{53,54}

Protection against NBD-Cl Inhibition of ATPase Activity by MgADP or \textit{P}_{i}. Panels A and B of Figure 4 show the MgADP protection data against NBD-Cl in wild-type and membrane-bound \textit{F}_{1}\textit{F}_{0} enzymes. It is seen that wild-type and mutant membranes were similarly protected against NBD-Cl inhibition. Earlier, we have shown that MgADP protects against NBD-Cl inhibition of wild-type soluble \textit{F}_{1} as well as membrane preparations of \textit{F}_{1}\textit{F}_{0}; however, protection occurred only at high concentrations (\textit{EC}_{50} ∼ 4.5 mM MgADP). In this study, the \textit{EC}_{50} values were 4.2, 3.1, 3.1, 4.4, 3.6, 2.5, and 4.8 mM for \textit{α}T349A, \textit{α}T349D, \textit{α}T349Q, \textit{α}T349R, \textit{β}R182A, \textit{α}T349R/\textit{β}R182A, and the wild type, respectively. We surmise that high concentrations are required to effectively keep the βE site occupied by MgADP in time average and thus hold back the access to NBD-Cl by sterically obstructing the site.\textsuperscript{19,29−35} This scheme is consistent with the conclusion of Orris et al.\textsuperscript{26} who provided X-ray crystallographic proof that NBD-Cl reacts specifically in the βE catalytic site. Therefore, we conclude that
NBD-Cl is reacting in $\beta$E in the mutants and that the ATPase activities measured in the mutants can be attributed to the ATP synthase enzyme and not to a contaminant.

Figure 5 shows the MgPi protection against the NBD-Cl reaction. It is obvious that Pi protected well against NBD-Cl inhibition of ATPase activity in the wild type, $\alpha_{T349R}$, and $\alpha_{T349R}/\beta_{R182A}$ but did not protect at all against NBD-Cl inactivation in $\alpha_{T349A}$, $\alpha_{T349D}$, $\alpha_{T349Q}$, $\beta_{R182A}$.

Inhibition of ATPase Activity by Fluoroaluminate, Fluoroscandium, and Azide. Subsequently, we examined the effects of transition state and ground state analogues. Panels A and B of Figure 6 show inhibition of wild-type and mutant enzymes by MgADP-fluoroaluminate and MgADP-fluoroscandium, respectively. The wild type was completely inhibited. Levels of AlF$_3$- and ScF$_3$-induced inhibition of mutants were $\sim$25 and $\sim$32% ($\alpha_{T349A}$), $\sim$5 and $\sim$12% ($\alpha_{T349D}$), $\sim$49 and $\sim$46% ($\alpha_{T349R}$), 17 and 22% ($\beta_{R182A}$), and $\sim$65 and $\sim$61% ($\alpha_{T349R}/\beta_{R182A}$), respectively. In contrast, mutant $\alpha_{T349Q}$ was particularly resistant to inhibition by either MgADP-fluoroaluminate or MgADP-fluoroscandium. Figure 6C shows that azide, another potent inhibitor of ATPase in ATP synthase, strongly inhibited the wild type but showed varied residual activity of $\sim$53% ($\alpha_{T349A}$), $\sim$90% ($\alpha_{T349D}$), $\sim$90% ($\alpha_{T349Q}$), $\sim$40% ($\alpha_{T349R}$), $\sim$72% ($\beta_{R182A}$), and $\sim$21% ($\alpha_{T349R}/\beta_{R182A}$) in mutants.

Inhibition of ATPase Activity by DCCD. Figure 7 shows the wild-type, $\alpha_{T349A}$, $\alpha_{T349D}$, $\alpha_{T349Q}$, $\alpha_{T349R}$, $\beta_{R182A}$, and $\alpha_{T349R}/\beta_{R182A}$ enzymes inactivated by DCCD. While the wild type is completely inhibited by 200 $\mu$M DCCD after incubation for 16 h at room temperature, mutants show varied degrees of inhibition. $\alpha_{T349A}$ is inhibited $\sim$31%, $\alpha_{T349Q}$ $\sim$7%, $\alpha_{T349R}$ $\sim$43%, $\beta_{R182A}$ $\sim$19%, and $\alpha_{T349R}/\beta_{R182A}$ $\sim$72%, while $\alpha_{T349D}$ is not inhibited at all. In another set of experiments with 2 or 5 h incubations using the same DCCD.
concentrations and reaction conditions, we found that the wild type was still fully inhibited, \( \alpha T349A \), \( \alpha T349D \), \( \alpha T349Q \), \( \alpha T349R \), and \( \beta R182A \) showed no inhibition, but double mutant \( \alpha T349R/\beta R182A \) was inhibited maximally by 25% (2 h) and 55% (5 h).

**DISCUSSION**

The objective of this study was to examine the functional role(s) of residue \( \alpha \text{Thr}-349 \) of \( E. \ coli \) ATP synthase. This residue is part of the strongly conserved \( \alpha \)-subunit VISIT-DG sequence. The VISIT-DG sequence residues are located in the proximity of the \( \alpha \)-subunit—\( \beta \)-subunit interface bordering the \( P_i \)
Membrane-bound F₁Fₒ showed similar contents of F₁ bound Pi analogues (Figure 1). Pi binding is fundamental for that the side chain of residue the (which also contained SO₄⁻) also established that introduction of negative or positive charge was independent membrane preparations of each mutant. The variation are means of at least four different experiments using two or three independent membrane preparations of each mutant. The variation was ±10% between different experiments. 

binding pocket (Figure 1B). X-ray crystal structures of the AlF₃-inhibited enzyme as well as the AlF₄⁻inhibited enzyme (which also contained SO₄²⁻ in a second catalytic site) show that the side chain of residue αThr-349 is very close to these bound Pₐ analogues (Figure 1). Pₐ binding is fundamental for ATP synthesis by ATP synthase. Therefore, the process of Pₐ binding can divulge a wealth of information about ATP synthesis. Mutagenic analysis and molecular modulation of Pₐ binding residues are some of the best ways to examine and appreciate the functional role of residues in the catalytic site.

Earlier studies established that mutagenesis combined with the use of the Pₐ protection assay against NBD-Cl inhibition, as well as the use of inhibitory analogues, allowed the characterization of functional role(s) of residues suspected to be involved in Pₐ binding. From analysis of eight such catalytic site residues, we determined that five residues, namely, αArg-376, βArg-182, βArg-246, βLys-155, and αSer-347, are critical for Pₐ binding and form a triangular subdomain within the catalytic site. While four residues, αArg-376, βArg-182, βArg-246, and βLys-155, were directly involved in Pₐ binding, the fifth residue, αSer-347, supported Pₐ binding and transition state stabilization through its interaction with βArg-246 (and possibly with βArg-182, too) (Figure 1B). Earlier, we also established that introduction of negative or positive charge at this location resulted in strong alteration of Pₐ binding, indicating that negative charge within the triangular subdomain was an important determinant of Pₐ binding. Here we used the same approaches to study the αThr-349 residue.

Generation of the αT₃₄₉A, αT₃₄₉D, αT₃₄₉Q, αT₃₄₉R, βR₁₈₂A, or αT₃₄₉R/βR₁₈₂A mutant did not affect the assembly or structural integrity of the membrane ATP synthase. Membrane-bound F₅, F₈ showed similar contents of F₅ α- and β-subunits compared to the wild type. The αT₃₄₉A, αT₃₄₉D, αT₃₄₉Q, or βR₁₈₂A mutation caused severe loss of oxidative phosphorylation as judged by growth on succinate or limiting glucose medium. Also, strong inhibition of ATPase activity was observed along with abrogation of Pₐ binding. The αT₃₄₉R/βR₁₈₂A double mutant allowed Pₐ binding with substantial oxidative phosphorylation and ATPase activity. The αT₃₄₉R mutant was interesting for it has very little ATPase activity with no oxidative phosphorylation and still allowed Pₐ binding just like double mutant αT₃₄₉R/βR₁₈₂A (Table 1 and Figure 5).

Fluoroaluminate and fluoroscodium in combination with MgADP potently inhibit wild-type E. coli ATP synthase, and both are believed to mimic the chemical transition state. Transition state-like structures involving the bound MgADP–AlF₄⁻ complex have been seen in catalytic sites in ATP synthase by X-ray crystallography. MgADP-fluoroaluminate or MgADP-fluoroscodium failed to inhibit αT₃₄₉D and αT₃₄₉Q mutants, indicating strong destabilization of the transition state, while partial inhibition occurred in αT₃₄₉A, and βArg-182, αT₃₄₉R, and double mutant αT₃₄₉R/βR₁₈₂A, representative of the partial destabilization of the transition state (Figure 6A,B). These results are in agreement with the amount of oxidative phosphorylation and ATPase activity found in each of the mutants. Evidently, α-subunit VISIT-DG sequence residue αThr-349 is involved directly in the transition state and in catalysis and therefore should be considered as a sixth member of the group of Pₐ binding residues that make up the triangular Pₐ binding pocket.

All mutations affected the degree of inhibition by azide, with double mutant αT₃₄₉R/βR₁₈₂A reducing it substantially (by ~80%) and αT₃₄₉A (~47%), αT₃₄₉D (~10%), αT₃₄₉Q (~10%), αT₃₄₉R (~60%), and βR₁₈₂A (~28%) reducing it less severely (Figure 6C). An X-ray crystallographic study of azide-induced inhibition of ATP hydrolysis showed that azide inhibits ATP synthase by forming a tight binding MgADP–azide complex in βDP catalytic sites, which resembles that formed by MgADP-beryllium fluoride, and may therefore be considered an analogue of the MgATP ground state. In the MgADP–azide complex, the azide occupies a position equivalent to that of the γ-phosphate of MgATP. Thus, all mutants also had effects on substrate binding by virtue of an effect at the γ-P position.

DCCD inhibits wild-type E. coli F₁ by reacting with residue βGlu-192 and/or εAsp-61 with the latter predominating at lower DCCD concentrations and/or shorter incubation times. As expected, wild-type ATP synthase was inhibited almost 100%. The αT₃₄₉D mutant was not inhibited at all, and αT₃₄₉A was inhibited ~30%, αT₃₄₉Q ~7%, αT₃₄₉R ~43%, βArg-182 ~20%, and double mutant αT₃₄₉R/βArg-182 ~72% (Figure 7). Notably, at shorter incubation times, while double mutant αT₃₄₉R/βArg-182 showed substantial inhibition, all the single mutations resisted inhibition (see Results). The data therefore indicate that in the αT₃₄₉R/βArg-182 double mutant ATPase activity on F₅ is partly coupled to proton translocation in F₈ which explains why double mutant αT₃₄₉R/βArg-182 retains some growth on succinate and in limiting glucose (Table 1).

It is interesting to note here that Pₐ binding and release events have been shown to be directly linked to rotation of the central stalk in single-molecule experiments. Perturbation of the Pₐ binding site might well be anticipated to perturb the integrity of the link between Pₐ binding and rotation and manifest as uncoupling. Thus, the data for the αThr-349 mutation strongly suggest that the Thr-OH group is needed for transition state stabilization and Pₐ binding.
It is established that Arg residues occur frequently in Pi binding sites in proteins, therefore, varying the number of Arg residues in the Pi binding site of ATP synthase seemed to be a useful approach. Residue αThr-349 lies 2.68 and 4.38 Å, 2.86 and 4.01 Å, and 3.61 and 3.40 Å from known P, binding residues βArg-182 and αArg-376, respectively, in AlF$_4^−$, AlF$_3^−$, and SO$_4^{2−}$-containing catalytic sites (nearest atom distances).

Thus, one experimental approach we used was to introduce mutation αT349R into the wild-type background (with βArg-182) in the presence of the βR182A mutation. The location of residue αThr-349 at the end of the P$_i$ binding pocket across the catalytic α-subunit–β-subunit interface with its side chain pointing toward the bound P$_i$ analogues also appeared to be a suitable location for the introduction of a new Arg. Apparently, the αT349R mutation would place extra positive charge relatively close to P$_i$ and double mutation αT349R/βR182A will allow the αT349R mutant Arg to fit into the large “hole” generated by the βAla-182 mutation. The βR182A mutant did not show P$_i$ binding, but the αT349R mutation “rescued” P$_i$ binding in combination with βR182A (Figure 5). On the basis of the loss of oxidative phosphorylation that was made evident by growth on succinate or limiting glucose medium along with very low ATPase activity, αArg-349 could be expected to assume the same exact stereochemical interactions achieved by βArg-182. Thus, electrostatic interaction per se is therefore important, and we conclude that the presence of at least one positive charge at this general location is a requisite determinant of initial P$_i$ binding in catalytic site βE. In addition, the αT349R mutation in the wild-type background totaling one extra positive charge did not prevent P$_i$ binding (Figure 5), but the presence of negative charge in the form of αT349D resulted in abrogation of P$_i$ binding. Presumably, the presence of Asp negated the positive charge of nearby residue βArg-182, resulting in the abrogation of P$_i$ binding.

αThr-349 is positioned close to bound AlF$_4^−$ in catalytic sites (see Figure 1B). The Thr-OH lies 5.46 Å from the F$_3$ atom in AlF$_4^−$ and thus may contribute to transition state stabilization by direct interaction. It may be mentioned that a similar conclusion was reached regarding the OH group contributed by αSer-347 of the E. coli VISIT-DG sequence and Ser-OH of the highly conserved “LSGGQ” ABC signature sequence in P-glycoprotein. Considering how P$_i$ binding is affected, αThr-349-OH lies 6.44 Å from O2 in SO$_4^{2−}$ and 5.56 Å from F1 of AlF$_3$ in the respective catalytic sites. Thus, some direct interaction may be operative. However, more important than the findings described above may be the fact that the Thr-OH lies 2.86 Å from NH2 of βArg-182 (in the AlF$_4^−$ site) and 2.68 Å from NH2 of βArg-182 in the AlF$_3$-occupied site. βArg-182 is strongly conserved and critical for P$_i$ binding and transition state stabilization. Further, the carbonyl O of αThr-349 lies 3.40 and 3.63 Å from NH1 and NH2, respectively, of αArg-376, another P$_i$ binding residue. The likely H-bond interaction between αThr-349 and βArg-182 (and αArg-376) suggests these residues act together to support P$_i$ binding and transition state stabilization.

In summary, the αThr-349 residue of the conserved VISIT-DG sequence in the ATP synthase α-subunit is required for catalysis, P$_i$ binding, and transition state stabilization. Introduction of Arg at this site can compensate for the absence of Arg at the known P$_i$ binding residue βArg-182 site. Furthermore, arrangement of positive charges with respect to one another is of paramount importance for oxidative phosphorylation and P$_i$ binding.
The ATP synthase complex of Escherichia coli provides a direct probe of nucleotide binding: Maximal ATP hydrolysis occurs with three sites occupied.

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