

Role of α -Subunit VISIT-DG Sequence Residues Ser-347 and Gly-351 in the Catalytic Sites of *Escherichia coli* ATP Synthase*

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This paper describes the role of α -subunit VISIT-DG sequence residues α Ser-347 and α Gly-351 in catalytic sites of *Escherichia coli* F₁F_o ATP synthase. X-ray structures show the very highly conserved α -subunit VISIT-DG sequence in close proximity to the conserved phosphate-binding residues α Arg-376, β Arg-182, β Lys-155, and β Arg-246 in the phosphate-binding subdomain. Mutations α S347Q and α G351Q caused loss of oxidative phosphorylation and reduced ATPase activity of F₁F_o in membranes by 100- and 150-fold, respectively, whereas α S347A mutation showed only a 13-fold loss of activity and also retained some oxidative phosphorylation activity. The ATPase of α S347Q mutant was not inhibited, and the α S347A mutant was slightly inhibited by MgADP-azide, MgADP-fluoroaluminate, or MgADP-fluoroscandium, in contrast to wild type and α G351Q mutant. Whereas 7-chloro-4-nitrobenzo-2-oxa-1,3-diazole (NBD-Cl) inhibited wild type and α G351Q mutant ATPase essentially completely, ATPase in α S347A or α S347Q mutant was inhibited maximally by ~80–90%, although reaction still occurred at residue β Tyr-297, proximal to the α -subunit VISIT-DG sequence, near the phosphate-binding pocket. Inhibition characteristics supported the conclusion that NBD-Cl reacts in β E (empty) catalytic sites, as shown previously by x-ray structure analysis. Phosphate protected against NBD-Cl inhibition in wild type and α G351Q mutant but not in α S347Q or α S347A mutant. The results demonstrate that α Ser-347 is an additional residue involved in phosphate-binding and transition state stabilization in ATP synthase catalytic sites. In contrast, α Gly-351, although strongly conserved and clearly important for function, appears not to play a direct role.

F₁F_o-ATP synthase is the enzyme responsible for ATP synthesis by oxidative or photophosphorylation in membranes of bacteria, mitochondria, and chloroplasts. It is the fundamental means of cell energy production in animals, plants, and almost all microorganisms. It works like a nanomotor and is structurally similar in all species. In its simplest form, as in *Escherichia coli*, it contains eight different subunits distributed in the water-soluble F₁ sector (subunits $\alpha_3\beta_3\gamma\delta\epsilon$) and the membrane-associated F_o sector (subunits ab_2c_{10}). The total molecular size

is ~530 kDa. In chloroplasts there are two isoforms of subunit *b*. In mitochondria, there are 7–9 additional subunits, depending on the source, but *in toto* they contribute only a small fraction of additional mass and may have regulatory roles (1–4).

ATP hydrolysis and synthesis occur in the F₁ sector. X-ray structures of bovine enzyme (5) established the presence of three catalytic sites at α/β subunit interfaces of the $\alpha_3\beta_3$ hexamer. Proton transport occurs through the membrane-embedded F_o. The γ -subunit contains three α -helices. Two of these helices form a coiled coil and are located in the central space of the $\alpha_3\beta_3$ hexamer. Proton gradient-driven clockwise rotation of γ (as viewed from the membrane) leads to ATP synthesis and anticlockwise rotation of γ results from ATP hydrolysis. In recent terminology, the rotor consists of $\gamma\epsilon c_n$, and the stator consists of $b_2\delta$ (6, 7). The function of the stator is to prevent co-rotation of catalytic sites with the rotor. Detailed reviews of ATP synthase structure and function may be found in Refs. 8–13.

To better understand the reaction mechanism of ATP synthesis and hydrolysis and their relationship to mechanical rotation in this biological nanomotor, we have focused our efforts on determining the role of conserved residues in and around catalytic site P_i-binding subdomain. Knowledge of P_i-binding residues and residues surrounding the P_i-binding subdomain is imperative for accomplishing (i) the molecular modulation of the catalytic site for the improved catalytic and motor function of this enzyme, (ii) an explanation of how ATP synthase binds ADP and P_i within its catalytic sites in the face of a relatively high ATP/ADP concentration ratio, and (iii) understanding the relationship between P_i binding and subunit rotation (14–16). Earlier attempts to measure P_i binding in purified *E. coli* F₁ using [³²P]P_i (15) or by competition with ATP or AMP-PNP² in fluorescence assays of nucleotide binding (18, 19) failed to detect appreciable P_i binding at physiological P_i concentration. So, we turned to the assay devised by Perez *et al.* (20) in which the protection afforded by P_i against inhibition of ATPase activity induced by covalent reaction with 7-chloro-4-nitrobenzo-2-oxa-1,3-diazole (NBD-Cl) provides the measure of P_i binding. Earlier Orriss *et al.* (21) showed by x-ray crystallography that the covalent adduct formed by NBD-Cl is specifically in the β E catalytic site (Fig. 1A); thus protection afforded by P_i indicates that binding of P_i occurs at the β E catalytic site. By modifying the above assay for use with *E. coli* purified F₁ or F₁F_o mem-

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² The abbreviations used are: AMP-PNP, 5'-adenylyl- β , γ -imidodiphosphate; NBD-Cl, 7-chloro-4-nitrobenzo-2-oxa-1,3-diazole; DTT, dithiothreitol; TES, 2-[[2-hydroxy-1,1-bis(hydroxymethyl)ethyl]amino]ethanesulfonic acid; DCCD, dicyclohexylcarbodiimide.

Role of α Ser-347 and α Gly-351 in *E. coli* ATP Synthase

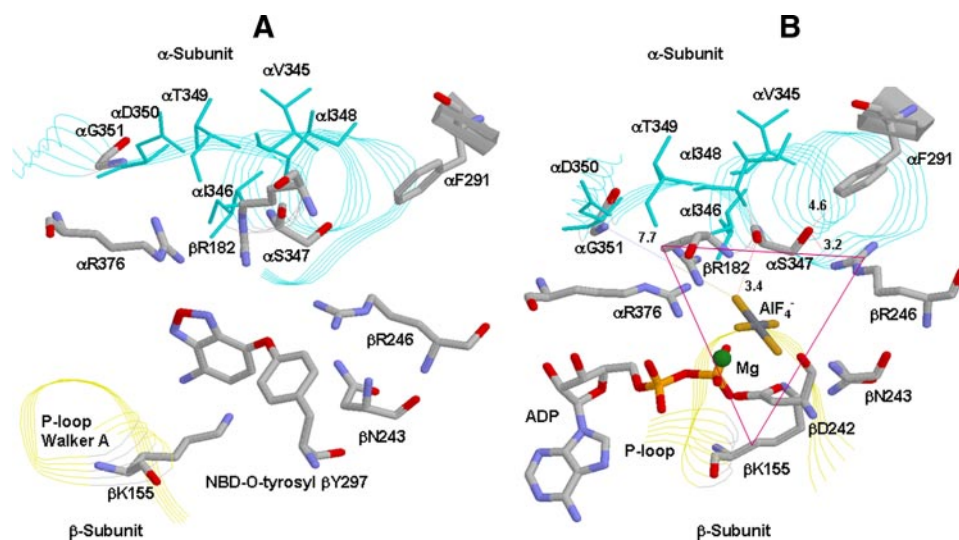


FIGURE 1. X-ray structures of catalytic sites in mitochondrial ATP synthase showing spatial relationship of α -subunit VISIT-DG sequence α S347 and α G351. A, reacted NBD-O-tyrosyl-297 in the β E site (21). B, the β DP site in the AlF_4^- -inhibited enzyme (23). *E. coli* residue numbering is shown. The triangle shows the residues β Lys-155, β Arg-182, β Arg-246, α Arg-376, and α Ser-347 forming a triangular P_i -binding site. Rasmol software was used to generate these figures.

<i>E. coli</i>	RAARVNAEYVEAFTKGEVKGKTGSLTALPIIETQAGDVSAFVPTNVI SITDGQ IFLETEL
<i>B. taurus</i>	RAAKMNDAF-----GGGSLTALPVIETQAGDVSAIYIPTNVI SITDGQ IFLETEL
<i>H. sapiens</i>	RAAKMNDAF-----GGGSLTALPVIETQAGDVSAIYIPTNVI SITDGQ IFLETEL
<i>P. abelii</i>	RAAKMNDAF-----GGGSLTALPVIETQAGDVSAIYIPTNVI SITDGQ IFLETEL
<i>D. rerio</i>	RAAKMNDNF-----GGGSLTALPVIETQAGDVSAIYIPTNVI SITDGQ IFLETEL
<i>R. norvegica</i>	RAAKMNDNF-----GGGSLTALPVIETQAGDVSAIYIPTNVI SITDGQ IFLETEL
<i>S. salar</i>	RAAKMNDNF-----GGGSLTALPVIETQAGDVSAIYIPTNVI SITDGQ IFLETEL
<i>G. gallus</i>	RAAKMNDNF-----GGGSLTALPAIETQAGDVSAIYIPTNVI SITDGQ IFLETEL
<i>X. tropicalis</i>	RAAKMNDHF-----GGGSLTALPVIETQAGDVSAIYIPTNVI SITDGQ IFLETEL
<i>A. aegypti</i>	RAAKMNDNF-----GGGSLTALPVIETQAGDVSAIYIPTNVI SITDGQ IFLETEL
<i>B. mori</i>	RAAKMNDNF-----GGGSLTALPVIETQAGDVSAIYIPTNVI SITDGQ IFLETEL
<i>B. malayi</i>	RAAKMNDNF-----GGGSLTALPVIETQAGDVSAIYIPTNVI SITDGQ IFLETEL
<i>P. fucata</i>	RAAKMNDNF-----GGGSLTALPVIETQAGDVSAIYIPTNVI SITDGQ IFLETEL
<i>S. purpuratus</i>	RAAKMNDNF-----GGGSLTALPVIETQAGDVSAIYIPTNVI SITDGQ IFLETEL
<i>C. intestinalis</i>	RAAKMNDNF-----GGGSLTALPVIETQAGDVSAIYIPTNVI SITDGQ IFLETEL
<i>H. mealybug</i>	RAAKMNDNF-----GGGSLTALPVIETQAGDVSAIYIPTNVI SITDGQ IFLETEL
<i>A. fumigatus</i>	RAAKMNDNF-----GGGSLTALPVIETQAGDVSAIYIPTNVI SITDGQ IFLETEL
<i>P. marneffei</i>	RAAKMNDNF-----GGGSLTALPVIETQAGDVSAIYIPTNVI SITDGQ IFLETEL
<i>S. cerevisiae</i>	RAAKMNDNF-----GGGSLTALPVIETQAGDVSAIYIPTNVI SITDGQ IFLETEL

FIGURE 2. Amino acid sequence alignment of evolutionarily conserved α -subunit VISIT-DG sequence. α -Subunit sequence from different species is aligned. The highly conserved VISIT-DG sequence is highlighted in gray. The starting residue arginine shown here for *E. coli* is α R300. Conserved α Ser-347 and α Gly-351 are denoted by bold font.

branes, we have previously investigated the relationship between P_i binding and catalysis for six residues, namely β Arg-246, β Asn-243, α Arg-376, β Lys-155, β Arg-182, and α Phe-291.³ All of these residues are positioned in proximity to the phosphate analogs AlF_3 or SO_4^{2-} in x-ray structures of catalytic sites (22, 23). We found that four residues, namely β Arg-246, α Arg-376, β Lys-155, and β Arg-182, grouped in a triangular fashion are directly involved in P_i binding (Fig. 1B) (24–30).

It is interesting to note that Penefsky (31) detected [^{32}P] P_i binding with a $K_d(\text{P}_i)$ in the range of 0.1 mM in mitochondrial membranes using a pressure ultrafiltration method, and the results are in agreement with data obtained from the NBD-Cl protection assay (20). However, Penefsky could not detect P_i binding in *E. coli* F_1F_o , and thus it is evident that P_i dissociates more rapidly from *E. coli* F_1 than it does from mitochondrial F_1 .

³ *E. coli* residue numbers are used throughout.

This unfortunately renders the potentially more convenient centrifuge column assay unsuitable with the *E. coli* enzyme.

A mechanism of condensation of P_i with MgADP was proposed by Senior *et al.* (32). The x-ray crystallography structure of bovine ATP synthase by Menz *et al.* (23) shows the transition state analog MgADP- AlF_4^- trapped in catalytic sites (Fig. 1B). It is clear from the geometry of this complex that the fluoroaluminate group occupies the position of the ATP- γ -phosphate in the predicted transition state. Similarly, Pedersen and co-workers (33) reported the first transition state-like structure of F_1 using enzyme obtained from rat liver and crystallized with the P_i analog vanadate (V_i). This work further demonstrated that ADP was not essential, suggesting that the MgVi-F_1 complex inhibited the catalytic activity to the same extent as that observed for the MgADP- Vi-F_1 complex. Unfortunately, neither MgVi nor MgADP- Vi inhibits the *E. coli* enzyme (24). Thus we have relied on inhibition of ATPase activity by fluoroaluminate (or fluoroscandium) to assess the potential to stabilize a transition state complex (24–26, 28, 30). Through mutagenesis and by employing the NBD-Cl protection assay as well as ATPase inhibition by transition state analogs, we can probe the direct or indirect role of residues in P_i binding. In this manuscript, we explore the possible role

played by α Ser-347 and α Gly-351 residues in the highly conserved α -subunit VISIT-DG sequence. Fig. 1B shows the location of α Ser-347 and α Gly-351 residues. Notably, α Ser-347 appears to occupy a strategic position in the P_i -binding subdomain. Fig. 2 shows the evolutionarily conserved α -subunit VISIT-DG sequence along with surrounding residues of α -subunit from a variety of species. The basic questions we asked were: what role does α Ser-347 or α Gly-351 play? Do the mutations α S347A, α S347Q, or α G351Q have any effect on P_i binding or transition state formation?

MATERIALS AND METHODS

Construction of Wild Type and Mutant Strains of *E. coli*—The wild type strain was pBWU13.4/DK8 (34). Mutagenesis was by the method of Vandeyar *et al.* (35). The template for oligonucleotide-directed mutagenesis was M13mp18 containing the HindIII-XbaI fragment from pSN6. pSN6 is a plasmid

containing the β Y331W mutation from plasmid pSWM4 (36) introduced on a SacI-EagI fragment into pBWU13.4 (34), which expresses all the ATP synthase genes. pSWM67/AN888 strain was used for α S347A mutant (37). The mutagenic oligonucleotide for α S347Q was CCAACGTAATCCAGATTACCGATGG, where the underlined bases introduce the mutation and a new XcmI restriction site, and that for α G351Q was CCATTACCGATCAGCAAATCTTCCTGGAAACC, where the underlined bases introduce the mutation and a silent mutation removes BglII 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 a Csp451 (an isoschizomer of BstBI) and PmlI fragment generating the new plasmids pZA13 (α S347Q/ β Y331W) and pZA14 (α G351Q/ β Y331W). Each plasmid was transformed into strain DK8 (38) containing a deletion of ATP synthase genes for expression of the mutant enzymes. It may be noted that both mutant strains contained the β Y331W mutation, which is valuable for measurement of nucleotide binding parameters (36) and does not affect function significantly on its own. Although the presence of β Y331W mutation was not utilized in this work, the Trp mutation was 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* membranes were prepared as in Ref. 39. 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*-aminobenzamide. 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*-aminobenzamide, 0.5 mM DTT, 0.5 mM EDTA. Prior to the experiments, the membranes were washed twice more by resuspension and ultracentrifugation in 50 mM TrisSO₄, pH 8.0, 2.5 mM MgSO₄. Growth yield in limiting glucose was measured as in Ref. 40. ATPase activity was measured in 1 ml of assay buffer containing 10 mM NaATP, 4 mM MgCl₂, 50 mM TrisSO₄, pH 8.5, at 37 °C. The reactions were started by the addition of membranes and stopped by the addition of SDS to 3.3% final concentration. P_i released was assayed as in Ref. 41. For wild type membranes (20–30 μ g of protein), reaction times were 5–10 min. For mutant membranes (40–60 μ g of protein), reaction times were 30–50 min. All of the reactions were shown to be linear with time and protein concentration. SDS gel electrophoresis on 10% acrylamide gels was as in Ref. 42. Immunoblotting with rabbit polyclonal anti-F₁- α and anti-F₁- β antibodies was as in Ref. 43.

Inhibition of ATPase Activity by NBD-Cl and Protection by MgADP or P_i—NBD-Cl was prepared as a stock solution in dimethyl sulfoxide and protected from light. The membranes (0.2–0.5 mg/ml) were reacted with NBD-Cl for 60 min in the dark at room temperature in 50 mM TrisSO₄, pH 8.0, 2.5 mM MgSO₄, and then 50- μ l aliquots were transferred to 1 ml of ATPase assay buffer to determine ATPase activity. Where protection from NBD-Cl inhibition by ADP or P_i was determined, the membranes were preincubated 60 min with protecting agent at room temperature before the addition of NBD-Cl.

MgSO₄ was present, equimolar with ADP or P_i. Control samples containing the ligand without added NBD-Cl were included. Neither P_i (up to 50 mM) nor MgADP (up to 10 mM) had any inhibitory effect alone.

Reversal of NBD-Cl Inhibited ATPase Activity by DTT—For reversal of NBD-Cl inhibition by DTT, the membranes were first reacted with NBD-Cl (150 μ M) for 1 h at room temperature, and 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 Azide, Fluoroaluminate, or Fluoroscandium—Azide inhibition was measured by preincubating membranes with varied concentrations of sodium azide for 30 min. Then 1 ml of ATPase assay buffer was added to measure the activity. For measurements of fluoroaluminate or fluoroscandium inhibition, the membranes were incubated 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 presence of AlCl₃ or ScCl₃ added at varied concentration (see “Results”). 50- μ l aliquots were then added to 1 ml of ATPase assay buffer, and activity was measured as 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)—Covalent modification of wild type and mutant membrane was performed as described by Weber *et al.* (44). ATPase activity was measured by adding 1 ml of ATPase assay buffer containing 10 mM NaATP, 4 mM MgCl₂, 50 mM TrisSO₄, pH 8.5, at 37 °C to the 100- μ l aliquots of 16 h DCCD-modified ATP synthase.

RESULTS

Growth Properties of α S347Q, α S347A, and α G351Q Mutants of E. coli ATP Synthase—Three new mutants, α S347Q, α S347A, and α G351Q, were generated. These two residues were chosen for mutagenesis because of their strong conservation in the α -subunit VISIT-DG sequence and their close proximity to the P_i-binding pocket. The α S347A mutant was used to appreciate the role of Ser-OH side chain in P_i binding and transition state. α S347Q and α G351Q mutants were designed to understand the impact of larger side chain of Gln on α Ser-347 and α Gly-351.

Table 1 shows that introduction of Gln as α S347Q and α G351Q resulted in the loss of oxidative phosphorylation. Both mutations prevented growth on succinate-containing medium, and growth yields in limiting glucose medium were reduced close to those of the ATP synthase null control. α S347A mutant, on the other hand, resulted in partial loss of oxidative phosphorylation. Specific ATPase activities of membrane preparations containing mutant enzymes were compared with wild type and null control, and the values are shown in Table 1. α S347Q and α G351Q reduced the ATPase activity by 100–150-fold, whereas ATPase activity was reduced only 13-fold by α S347A. Membranes prepared from the mutants contained the same amount of α and β subunits as wild type, as determined by SDS gel electrophoresis and immunoblotting (26) (data not shown); therefore, reduced ATPase is not due to impaired

TABLE 1
Effects of α Ser-347 and α Gly-351 mutation on cell growth and ATPase activity

Mutation ^a	Growth on succinate ^b	Growth yield in limiting glucose	ATPase activity ^c
		%	$\mu\text{mol}/\text{min}/\text{mg}$
Wild type	++++	100	28
Null	—	46	0
β Y331W	++++	95	26
α S347Q	±	55	0.20
α S347A	+	65	2.18
α G351Q	±	54	0.25

^a Wild type, pBWU13.4/DK8; Null, pUC118/DK8, α S347Q/DK8, α G351Q/DK8, and α S347A/AN888. Both α S347Q and α G351Q mutants were expressed with the β Y331W mutation also present, which does not significantly affect growth. The data are the means of four to six experiments each.

^b Growth on succinate plates after 3 days estimated by eye. +++++, heavy growth; +, partial growth; ±, very light growth; —, no growth.

^c ATPase activity was measured at 37 °C and expressed as μmol of ATP hydrolyzed/min/mg of membrane protein. Each individual experimental point is itself the mean of duplicate assay tubes. The data are derived from two separate membrane preparations. The results from separate membrane preparations were in excellent agreement within $\pm 10\%$.

assembly of ATP synthase or loss of F_1 during membrane preparation.

Inhibition of ATPase Activity of ATP Synthase in Membranes by NBD-Cl and Reversal by Dithiothreitol—We previously established that P_i binding by mutant or wild type ATP synthase can be assayed using either membrane preparations or purified F_1 , with equivalent results (24, 26). In this work we used membrane preparations that are more convenient. Fig. 3 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 with no residual activity, and this is consistent with previous studies (24–28, 30). α G351Q mutant was also completely inhibited, and α S347Q or α S347A mutant were inhibited by ~ 80 – 90% with ~ 20 – 10% residual activity. In previous studies (24–28, 30), we have noted several instances where mutant ATP synthases were incompletely inhibited by NBD-Cl. To be sure that maximal reaction with NBD-Cl had been reached, we incubated each membrane preparation with $150 \mu\text{M}$ NBD-Cl for 1 h as in Fig. 3, followed by an additional pulse of $200 \mu\text{M}$ NBD-Cl, continuing the incubation for an additional hour before assaying ATPase activity. Very little or no additional inhibition occurred (Fig. 4, left panel). This shows that the reaction of NBD-Cl was complete and that fully reacted α S347Q mutant membranes retained residual activity. Next, we checked whether inactivation by NBD-Cl could be reversed by the addition of the reducing agent DTT because reversibility by DTT was indicative of specificity of reaction in previous studies. Wild type and mutant enzymes were preincubated with $150 \mu\text{M}$ NBD-Cl as in Fig. 3, and then 4 mM DTT was added, and incubation continued for 1 h before ATPase assay. It was seen that DTT completely restored full activity in all cases (Fig. 4, right panel). This indicates that NBD-Cl reacts specifically with residue β Tyr-297 in the wild type as well as in both mutants (45, 46).

Protection against NBD-Cl Inhibition of ATPase Activity by MgADP or P_i —Fig. 5 shows the data for MgADP protection in membrane enzymes, and it is seen that wild type and mutants were protected against NBD-Cl inhibition. Previously we have shown that MgADP protects against NBD-Cl inhibition of wild type soluble F_1 as well as membrane preparations of F_1F_0 ; how-

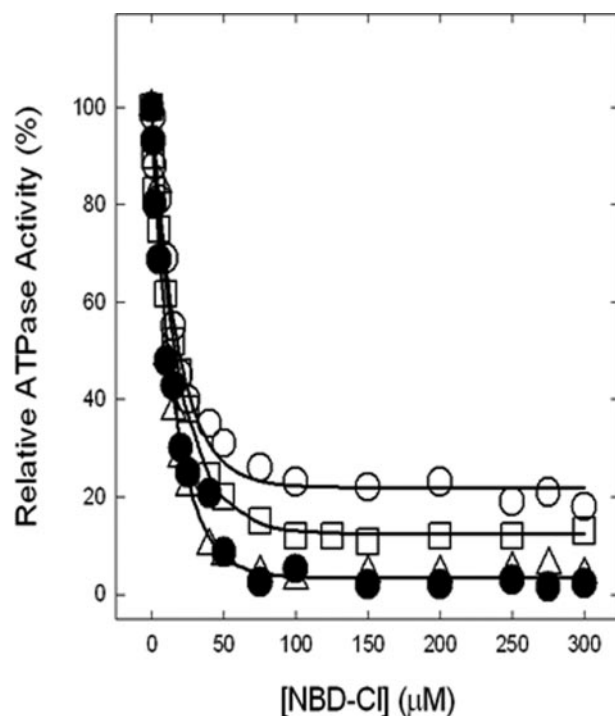


FIGURE 3. Inhibition of membrane-bound wild type and mutant ATP synthase by NBD-Cl. The membranes were preincubated for 60 min at room temperature with varied concentration of NBD-Cl, and then aliquots added to 1 ml of assay buffer and ATPase activity were determined. The details are given under "Materials and Methods." ●, wild type; ○, α S347A; □, α S347Q; △, α G351Q. Each data point represents an average of at least four experiments, using two independent membrane preparations of each mutant. The results agreed within $\pm 10\%$.

ever, protection occurred only at high concentrations ($EC_{50} = \sim 4.5 \text{ mM}$ MgADP). In this study the EC_{50} values were 5.1, 2.9, 3.0, and 4.0 mM for wild type, α S347Q, α S347A, and α G351Q, respectively. We reason that high concentrations are required to effectively keep the βE site occupied by MgADP in time average and thus impede the access to NBD-Cl by sterically obstructing the site (24–30). This idea is consistent with the conclusion of Orriss *et al.* (21), who provided evidence that NBD-Cl reacts specifically in the βE catalytic site by x-ray crystallographic studies. We conclude that NBD-Cl is reacting in βE in the mutants and that the ATPase activities measured in the mutants are referable to ATP synthase enzyme and not caused by a contaminant.

Mg P_i protection against NBD-Cl reaction is presented in Fig. 6. It is evident that P_i protected well against NBD-Cl inhibition of ATPase activity in wild type and α G351Q mutant but did not protect at all against inactivation in α S347Q or α S347A mutants.

Inhibition of ATPase Activity by Fluoroaluminate, Fluoroscandium, and Azide—We next examined the effects of transition state and ground state analogs. Fig. 7 (A and B) show inhibition of wild type and mutant enzymes by MgADP-fluoroaluminate and MgADP-fluoroscandium, respectively. Wild type and α G351Q were completely inhibited. α S347A showed only $\sim 25\%$ inhibition. In contrast, the mutant α S347Q was remarkably resistant to inhibition. Azide is also a potent inhibitor of ATPase in ATP synthase. Fig. 7C shows that although wild type is strongly inhibited by azide, the mutants showed

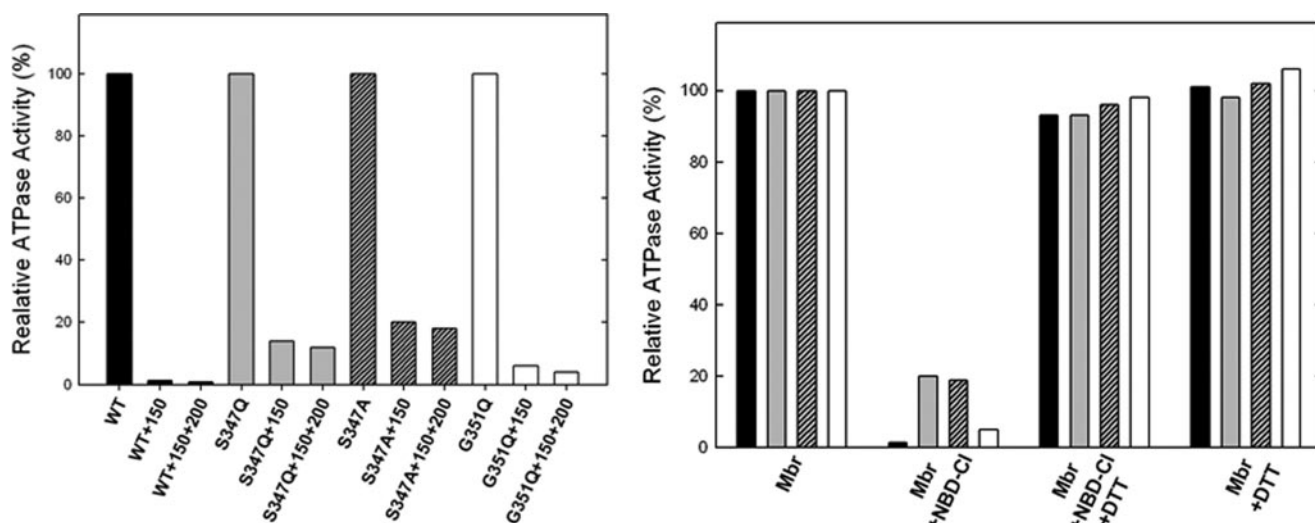


FIGURE 4. Results of extra pulse of NBD-Cl in mutants and reversal of NBD-Cl effects by DTT. *Left panel*, membrane ATP synthase was inhibited with 150 μ M NBD-Cl for 60 min under conditions as described in Fig. 3. Then a further pulse of 200 μ M NBD-Cl was added, and incubation continued for 1 h before assay. *Right panel*, membrane ATP synthase (*Mbr*) was incubated with or without 150 μ M NBD-Cl for 60 min under conditions as described for Fig. 3. The degree of inhibition was assayed. In parallel samples, 4 mM DTT was then added, and incubation continued for further 60 min before assay. Each set of bars represents wild type, α S347Q, α S347A, and α G351Q from left to right. The absolute residual ATPase activity values are as follows: wild type, 28, 0.36, 0.22; α S347Q, 0.20, 0.03, 0.02; α S347A, 2.18, 0.44, 0.39; and α G351Q, 0.25, 0.02, 0.01.

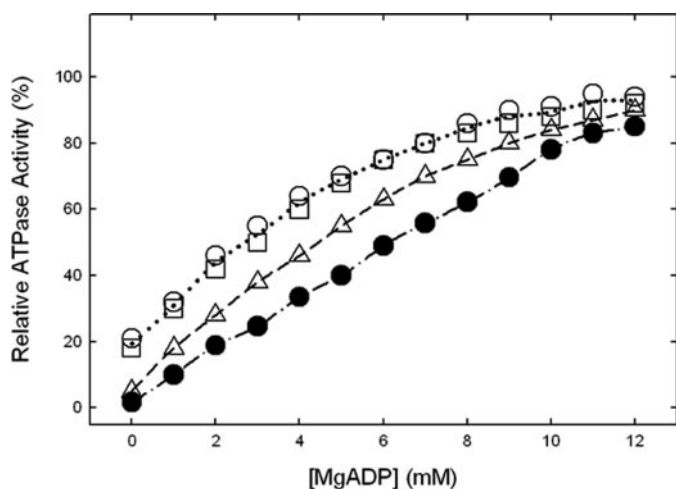


FIGURE 5. Protection against NBD-Cl reaction by MgADP. Wild type and mutant membrane were preincubated for 1 h at room temperature with varied concentrations of MgADP as shown, then 150 μ M NBD-Cl was added, and incubation continued at room temperature in the dark for 1 h. The aliquots were then assayed for ATPase activity. ●, wild type; ○, α S347A; □, α S347Q; △, α G351Q. The results are the means of quadruplicate experiments which agreed within $\pm 10\%$.

varied resistance with $\sim 70\%$ inhibition in α G351Q and only ~ 20 – 25% inhibition in α S347Q and α S347A mutants.

Inhibition of ATPase Activity by DCCD—Fig. 8 shows the wild type and α S347Q, α S347A, and α G351Q mutant enzymes inactivated by DCCD. Although wild type is completely inhibited by 200 μ M DCCD after 16 h of incubation at room temperature, mutants show varied degrees of inhibition. α G351Q is inhibited about 10%, α S347A is inhibited only $\sim 30\%$, and α S347Q is not inhibited at all. In a similar series of experiments, carried out with the same range of DCCD concentrations and reaction conditions, but for only 2- or 5-h incubations, we found that wild type still became fully inhibited, α G351Q and α S347Q both showed zero inhibition, and α S347A was inhibited maximally by 6% (2 h) and 15% (5 h).

DISCUSSION

The goal of this study was to examine the functional role(s) of residue α Ser-347 and α Gly-351 of *E. coli* ATP synthase. These residues are part of the strongly conserved α -subunit VISIT-DG sequence. The VISIT-DG sequence residues are located in close proximity to the α/β interface flanking the P_i -binding pocket (Fig. 1B). X-ray crystal structures of the AlF_3 -inhibited enzyme (22) as well as the AlF_4^- -inhibited enzyme (which also contained SO_4^{2-} in a second catalytic site) (23) show that the side chain of residue α Ser-347 is very close to these bound P_i analogs (Fig. 1) and that α Gly-351 is also close. P_i binding is a primary step in ATP synthesis by ATP synthase, thus exploring the molecular basis of P_i binding is an important way to examine and understand the functional role of residues in the catalytic site.

Earlier studies established that mutagenesis combined with the use of the P_i protection assay against NBD-Cl inhibition, as well as the use of inhibitory analogs, enabled characterization of functional role(s) of residues suspected to be involved in P_i binding (24–30). From analysis of six such catalytic site residues, we determined that four residues, namely, α Arg-376, β Arg-182, β Arg-246, and β Lys-155, are critical for P_i binding and form a triangular subdomain within the catalytic site (24–30) (Fig. 1B). We also established that introduction of a negative or positive charge in this location resulted in drastic modulation of P_i binding (25, 26, 30), indicating that negative charge within the triangular subdomain was an important determinant of P_i binding. Here we used the same approaches to study residues α Ser-347 and α Gly-351.

We introduced the mutations α S347Q, α S347A, and α G351Q, none of which affected assembly nor structural integrity of the membrane ATP synthase. Membranes showed similar content of F_1 - α and β subunits as compared with wild type. Both α S347Q or α G351Q mutations had severely inhibitory effects on oxidative phosphorylation as judged by growth on

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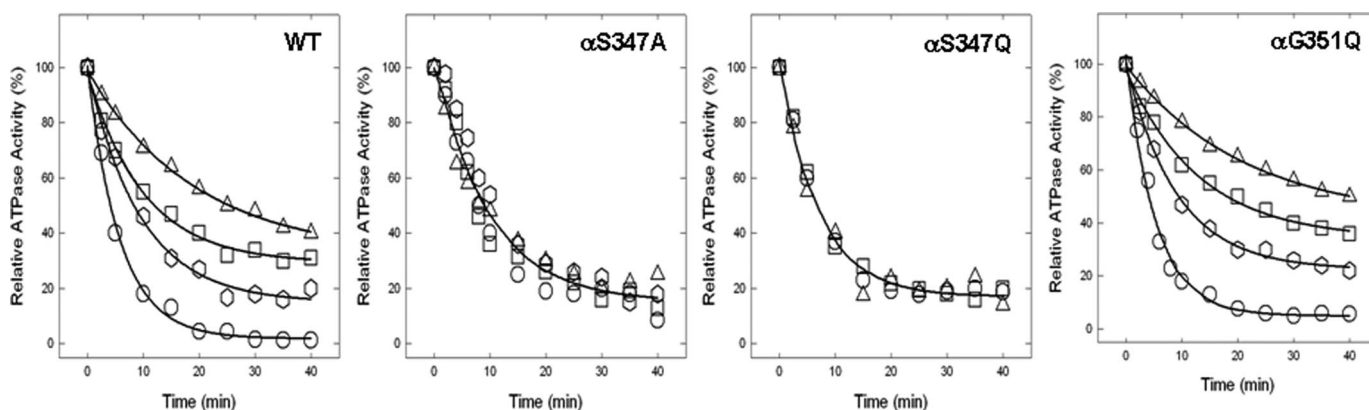


FIGURE 6. **Protection by P_i of ATPase activity in wild type (WT) and mutant membranes from inactivation by NBD-Cl.** The membranes were preincubated with MgP_i at 0, 2.5, 5, or 10 mM concentration as shown, for 60 min at room temperature. Then NBD-Cl ($150 \mu M$) was added, and aliquots were withdrawn for assay at time intervals as shown. ATPase activity remaining is plotted against time of incubation with NBD-Cl. \circ , no P_i added; \square , 2.5 mM P_i ; \triangle , 5 mM P_i ; \diamond , 10 mM P_i . Each data point represents the average of four different experiments using two independent membrane preparations of each mutant.

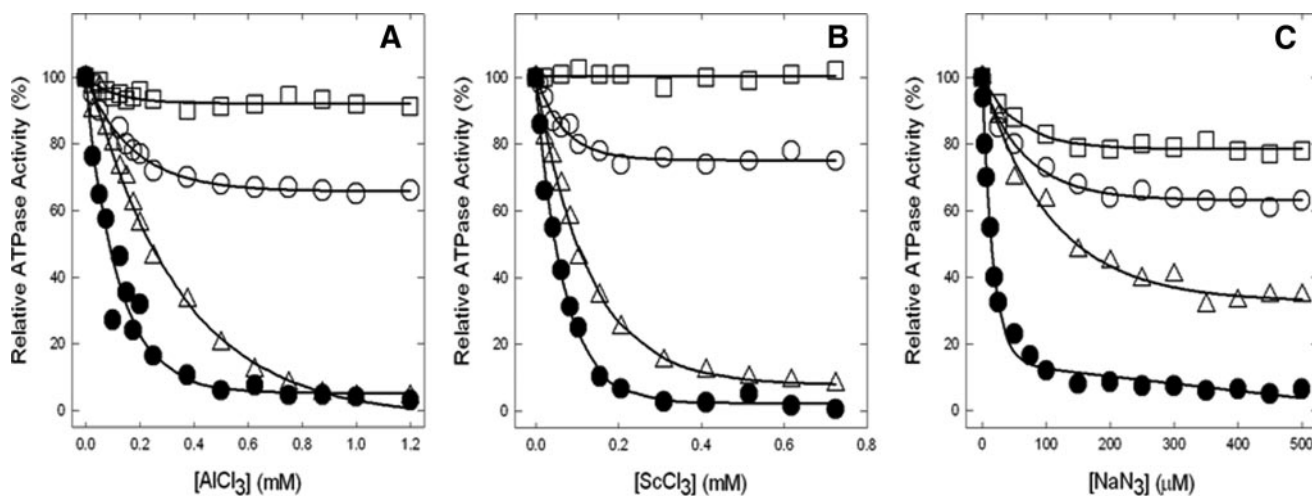


FIGURE 7. **Inhibition of membrane ATPase activity from mutant and wild type ATP synthase enzymes by fluoroaluminate, fluoroscandium, and azide.** The membranes were preincubated for 60 min at room temperature with 1 mM $MgADP$, 10 mM NaF , and the indicated concentrations of $AlCl_3$ (A) or $ScCl_3$ (B). Then aliquots were added to 1 ml of assay buffer, and ATPase activity was determined. Sodium azide was added directly to the membranes and incubated for 30 min before assay (C), for details see "Materials and Methods." \bullet , wild type; \circ , $\alpha S347A$; \square , $\alpha S347Q$; \triangle , $\alpha G351Q$. All of the data points are the means of at least quadruplicate experiments. The variation was $\pm 10\%$ between different experiments.

succinate or limiting glucose medium, and both strongly inhibited ATPase activity. On the other hand the $\alpha S347A$ mutation showed small residual oxidative phosphorylation and ATPase activity (Table 1). The results with the $\alpha S347Q$ and $\alpha S347A$ mutants showed that they abolished P_i binding (Fig. 6). Although based on Table 1 data for $\alpha S347A$ mutant, it can be argued that there could be a small amount of P_i binding in cells but not significant enough to be measurable in the P_i binding assay of membranes (Fig. 6). Fluoroaluminate and fluoroscandium in combination with $MgADP$ potently inhibit wild type *E. coli* ATP synthase (24–27, 30, 47, 48), and both are believed to mimic the chemical transition state. Transition state-like structures involving bound $MgADP-AlF_4^-$ complex have been seen in catalytic sites in ATP synthase by x-ray crystallography (23). It was evident that the $\alpha S347Q$ mutant strongly destabilized the transition state (Fig. 7, A and B), because no inhibition by $MgADP$ -fluoroaluminate or $MgADP$ -fluoroscandium was apparent. Clearly, therefore, residue α Ser-347 is involved directly and to an important degree in catalysis and may be added as a

fifth member of the group of P_i -binding residues that make up the triangular P_i -binding pocket. $\alpha S347A$ mutant did show some residual inhibition ($\sim 25\%$) with both $MgADP$ -fluoroaluminate and $MgADP$ -fluoroscandium, which is in agreement with the partial oxidative phosphorylation and ATPase activity found in this mutant. In contrast, the $\alpha G351Q$ mutation did not prevent P_i binding (Fig. 6) and had lesser effects in destabilizing the transition state as judged by fluoroaluminate and fluoroscandium inhibition of ATPase (Fig. 7, A and B). Its effects on catalysis are therefore more indirect.

All of the mutations affected the degree of inhibition by azide, with $\alpha S347Q$ reducing it substantially (by $\sim 80\%$), $\alpha S347A$ reducing it substantially ($\sim 75\%$), and $\alpha G351Q$ reducing it by $\sim 30\%$ (Fig. 7C). A recent x-ray crystallography study (49) showed that azide inhibits ATP synthase by forming a tightly binding $MgADP$ -azide complex in βDP catalytic sites, which resembles that formed by $MgADP$ -beryllium fluoride and may therefore be considered an analog of the $MgATP$ ground state. In the $MgADP$ -azide complex, azide occupies a

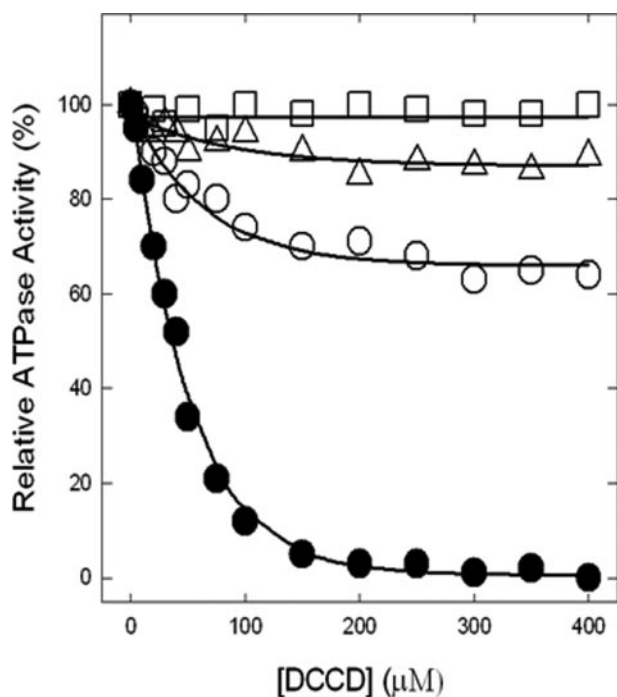


FIGURE 8. Inhibition of membrane ATPase activity from mutant and wild type ATP synthase enzymes by DCCD. The membranes were preincubated for 16 h at room temperature with varied concentrations of DCCD indicated in the figure. Then 1 ml of ATPase assay buffer was added to determine the activity. ●, wild type; ○, α S347A; □, α S347Q; △, α G351Q. All of the data points are means of at least quadruplicate experiments. The variation was $\pm 10\%$ between different experiments.

position equivalent to that of the γ -phosphate of MgATP. Thus mutants also had effects on substrate binding by virtue of an effect at the γ -phosphate position.

DCCD inhibits wild type *E. coli* F_1 by reacting with residue β Glu-192 (50) and/or c Asp-61 (51), with the latter predominating at lower DCCD concentration and/or shorter incubation time. As expected, wild type ATP synthase was inhibited almost 100%. α S347Q mutant was not inhibited at all, α G351Q was inhibited to $\sim 10\%$, and mutant α S347A is inhibited $\sim 30\%$ (Fig. 8). Notably, at shorter incubation times, α S347A showed even less inhibition (see "Results"). The data therefore indicate that in the α S347A mutant, ATPase activity on F_1 is only partly coupled to proton translocation in F_0 , which explains why α S347A mutant retains some growth on succinate and in limiting glucose (Table 1). It is interesting to note here that P_i binding and release events have been shown to be directly linked to rotation of the central stalk in single molecule experiments (52). Perturbation of the P_i -binding site might well be anticipated to perturb the integrity of the link between P_i binding and rotation and be manifested as uncoupling. The overall data on α S347A mutant strongly suggests that the Ser-OH group is needed for transition state stabilization and P_i binding.

The availability of x-ray structures allows one to discuss in detail the roles of residues α Ser-347 and α Gly-351. α Ser-347 is positioned close to bound AlF_4^- in catalytic sites (Fig. 1B) (23). The Ser-OH lies 5.0 Å from the F1 and F3 atoms in AlF_4^- and thus may contribute to transition state stabilization by direct interaction. It may be remarked that a similar conclusion was reached regarding the Ser-OH of the highly conserved LSGGQ

ABC signature sequence in P-glycoprotein (17). Considering how P_i binding is affected, α Ser-347-OH lies 6.1 Å from atom O_2 in SO_4^{2-} (23) and 4.6 Å from F1 of AlF_3 in the respective catalytic sites (22). Thus some direct interaction may be operative. However, more important than the above may be the fact that the Ser-OH lies 3.2 Å from the NH_2 of β Arg-246 (in the AlF_4^- site) and 3.0 and 4.1 Å, respectively from NH_2 and $NH1$ of β Arg-246 in the AlF_3 -occupied site. β Arg-246 is strongly conserved and critical for P_i binding and transition state stabilization (24). Further, the carbonyl-O of α Ser-347 lies 3.2 Å from NH_2 of β Arg-182, another P_i -binding residue. The likely H-bond interaction between α Ser-347 and β Arg-246 (and β Arg-182) suggests these residues act together to support P_i binding and transition state stabilization. α Gly-351 is located at a distance of 7.7 Å from AlF_4^- and 8.7 Å from SO_4^{2-} . A more indirect role in catalysis is therefore indicated, likely predominantly structural in nature.

In summary, both α Ser-347 and α Gly-351 of the conserved VISIT-DG sequence in ATP synthase α -subunit are required for catalysis. α Ser-347 plays the more important role and is required for P_i binding and transition state stabilization.

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