Residues responsible for phosphate binding in F₁F₀-ATP synthase catalytic sites are of significant interest because phosphate binding is believed linked to proton gradient-driven subunit rotation. From x-ray structures, a phosphate-binding subdomain is evident in catalytic sites, with conserved βArg-246 in a suitable position to bind phosphate. Mutations βR246Q, βR246K, and βR246A in *Escherichia coli* were found to impair oxidative phosphorylation and to reduce ATPase activity of purified F₁ by 100-fold. In contrast to wild type, ATPase of mutants was not inhibited by MgADP-fluoroaluminate or MgADP-fluorescandium, showing the Arg side chain is required for wild-type transition state formation. Whereas 7-chloro-4-nitrobenzo-2-oxa-1,3-diazole (NBD-Cl) inhibited wild-type ATPase essentially completely, ATPase in mutants was inhibited maximally by ~50%, although reaction still occurred at residue βTyr-297, proximal to βArg-246 in the phosphate-binding pocket. Inhibition characteristics supported the conclusion that NBD-Cl reacts in βE (empty) catalytic sites, as shown previously by x-ray structure analysis. Phosphate protected against NBD-Cl inhibition in wild type but not in mutants. The results show that phosphate can bind in the βE catalytic site of *E. coli* F₁ and that βArg-246 is an important phosphate-binding residue.

ATP synthesis from ADP and Pᵢ in oxidative and photophosphorylation occurs in the catalytic sites of F₁F₀-ATP synthase. This membrane enzyme uses the energy of an ion gradient (usually protons) to drive rotation of a “rotor” composed of subunits. This membrane enzyme uses the energy of an ion gradient (usually protons) to drive rotation of a “rotor” composed of subunits with conserved βArg-246 in a suitable position to bind phosphate. Mutations βR246Q, βR246K, and βR246A in *Escherichia coli* were found to impair oxidative phosphorylation and to reduce ATPase activity of purified F₁ by 100-fold. In contrast to wild type, ATPase of mutants was not inhibited by MgADP-fluoroaluminate or MgADP-fluorescandium, showing the Arg side chain is required for wild-type transition state formation. Whereas 7-chloro-4-nitrobenzo-2-oxa-1,3-diazole (NBD-Cl) inhibited wild-type ATPase essentially completely, ATPase in mutants was inhibited maximally by ~50%, although reaction still occurred at residue βTyr-297, proximal to βArg-246 in the phosphate-binding pocket. Inhibition characteristics supported the conclusion that NBD-Cl reacts in βE (empty) catalytic sites, as shown previously by x-ray structure analysis. Phosphate protected against NBD-Cl inhibition in wild type but not in mutants. The results show that phosphate can bind in the βE catalytic site of *E. coli* F₁ and that βArg-246 is an important phosphate-binding residue.

Based on kinetic measurements, Boyer and co-workers (5, 6) hypothesized that the affinity of catalytic sites for Pᵢ is increased by the proton gradient, and currently the concept that Pᵢ binding is enhanced by proton gradient-driven subunit rotation appears well supported. Significant Pᵢ binding to *Escherichia coli* F₁ catalytic sites was not measurable by direct binding assay using radioactive Pᵢ (7). The Kᵢ value for Pᵢ binding was >10 mM as measured by competition with Mg-AMPPNP or ATP in fluorescence titrations using the βY331W mutant enzyme (8–10). Calculations based on unisite catalysis kinetics showed that KᵢPᵢ in unisite catalysis is 2.3 × 10⁻³ M in *E. coli* and 87 μM in mitochondrial F₁ (11). Although not directly applicable to physiological catalysis (2), such calculations underline the weak affinity that the catalytic site of highest affinity for ATP has for Pᵢ, in the absence of proton-driven rotation. On the other hand, measurements of KᵢPᵢ for ATP synthesis are in the range of ~1 mM (12–14). An important conclusion is that Pᵢ binding and release steps are likely to be intimately linked to rotational movements of subunits (1, 2, 15).

An often overlooked but crucial question in the mechanism of ATP synthesis is, how does the enzyme bind ADP plus Pᵢ, and not ATP into catalytic sites? In active cells the cytoplasmic concentrations of ATP and Pᵢ are approximately in the 2–5 mM range, whereas that of ADP is at least 10–50-fold lower. Equilibrium binding assays have established that both ADP and ATP bind to catalytic sites of purified F₁, and detergent-solubilized F₁F₀ with relatively similar binding affinities (8, 9, 16, 17). Obviously, the enzyme must have evolved a specific mechanism for selectively binding ADP into catalytic sites while contemporaneously discouraging access of ATP during proton-driven rotation and ATP synthesis. One hypothesis is that during ATP synthesis, proton gradient-driven rotation of subunits drives an empty catalytic site to bind Pᵢ tightly, thus stereochmica precluding ATP binding and therefore selectively favoring ADP binding (18). Further understanding of features that determine Pᵢ binding is therefore of major importance in formulating a mechanism for ATP synthesis. So far, none of the residues directly involved in binding Pᵢ has been identified experimentally.

X-ray structural analysis has indicated the possible location of the Pᵢ binding subdomain in the catalytic sites. A residue within this subdomain that could be significantly involved in Pᵢ binding is βArg-246 (equivalent to βArg-260 in mitochondrial F₁), conserved in all known species. Fig. 1A shows ADP-AlF₄⁻ bound in the βDP catalytic site (19) in what is believed to be a transition state analog structure. Fig. 1B shows the βDP site with ADP and AlF₄⁻ bound in what is believed to be a late transition state or early ground state structure (20), and Fig. 1C shows the βDP + Pᵢ site (also called the “half-closed” or...
The distance between the phosphate moiety and βArg-246 changes decrementally as the transition state collapses to the post-hydrolysis state. Moreover, in Fig. 1, A–C, residue βTyr-297 lies close to the phosphate moiety, at the end of what appears to be a phosphate-binding pocket also demarcated by residue βArg-246. The distance between the phosphate moiety and βArg-246 increases incrementally as the transition state collapses to the post-hydrolysis state. 

**Experimental Procedures**

**Preparation of E. coli Membranes; Purification of F$_1$; Measurement of Growth Yield in Limiting Glucose Medium; Assay of ATPase Activity of Membranes or Purified F$_1$—E. coli membranes were prepared as described previously (26). Prior to the experiments, membranes were washed twice by resuspension and ultracentrifugation in 50 mM TrisSO$_4$, pH 8.0, 2.5 mM MgSO$_4$. F$_1$ was purified as described previously (27). Prior to the experiments, F$_1$ samples (100 μl) were passed twice through 1-nl centrifuge columns (Sephadex G-50) equilibrated in 50 mM TrisSO$_4$, pH 8.0, 2.5 mM MgSO$_4$. Growth yield in limiting glucose was measured as described previously (28). ATPase activity was measured in 1 ml of assay buffer containing 10 mM NaATP, 4 mM MgCl$_2$, 50 mM TrisSO$_4$, pH 8.5. Specific activity was measured at 30 °C, and measurements of inhibition by inhibitors were done at room temperature. Reactions were started by addition of enzyme and stopped by addition of SDS to 3.3% final concentration. Pi released was assayed as described previously (29). For wild-type membranes and F$_1$, reaction times were 2–10 min. For mutant enzymes reaction times were 30–120 min. All reactions were shown to be linear with time and protein concentration.

**E. coli Strains—**For purified F$_1$, wild-type strain SWM1 was used (30), and for membranes wild type was pBWU13.4/DK8 (31). For fluorescence titrations with purified F$_1$, strain SWM4 (βY331W) (8) was used as the “wild type.” Mutant strains were βR246K/βY331W, βR246K/βY331A, and βR246A/βY331W, all three in DK8 and constructed as indicated below.

**Construction of Mutant Strains of E. coli—**pSN6 is a plasmid containing the βY331W mutation from plasmid pSWM4 (8) introduced on a SacI-EagI fragment into pBWU15.4/DK8 (31). For fluorescence titrations with purified F$_1$, strain SWM4 (βY331W) (8) was used as the “wild type.” Mutant strains were βR246K/βY331W, βR246K/βY331A, and βR246A/βY331W, all three in DK8 and constructed as indicated below.

**Construction of Mutant Strains of E. coli—**pSN6 is a plasmid containing the βY331W mutation from plasmid pSWM4 (8) introduced on a SacI-EagI fragment into pBWU15.4/DK8 (31) which expresses all the ATP synthase genes. The template for oligonucleotide-directed mutagenesis was M13mp18 containing the HindIII-XbaI fragment from pSN6. Mutagenesis was by the method of Vandeyar et al. (32). Mutagenic oligonucleotides were as follows: for βR246Q, GACAACATCTATAAGTATCCCTGCGCCGG, where the underlined bases introduce the mutation and a new BstXI restriction site; for βR246K, GACAACATCTATAQATACCTGGCGCCGG, where the underlined bases introduce the mutation and a new BstXI restriction site; and for βR246A, GACAACATCTATACCTGGCGCCGG, where the underlined bases introduce the mutation and a new BstXI restriction site; and for βR246A, GACAACATCTATACCTGGCGCCGG, where the underlined bases introduce the mutation site and a new NsiI 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 SacI-EagI fragments, generating the new plasmids pZA5 (βR246Q/βY331W), pZA6 (βR246K/βY331W), and pZA7 (βR246A/βY331W), respectively. Each plasmid was transformed into strain DK8 (33) containing a deletion of ATP synthase genes for expression of the mutant enzymes.

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Footnote:

2 The abbreviations used are: NBD-CI, 7-chloro-4-nitrobenzo-2-oxa-1,3-diazole; DTT, dithiothreitol; AMPPNP, adenosine 5’-β,γ-imino-triphosphate.
Arguments 246 in E. coli F1 ATPase Catalytic Sites

<table>
<thead>
<tr>
<th>Mutation</th>
<th>Growth on succinate</th>
<th>Growth yield in limiting glucose</th>
<th>ATPase activity of purified F1</th>
</tr>
</thead>
<tbody>
<tr>
<td>Wild-type</td>
<td>+/–</td>
<td>100</td>
<td>28</td>
</tr>
<tr>
<td>Null</td>
<td>–</td>
<td>42</td>
<td></td>
</tr>
<tr>
<td>βY331W alone</td>
<td>+/–</td>
<td>93</td>
<td>14</td>
</tr>
<tr>
<td>βR246Q</td>
<td>–</td>
<td>45</td>
<td>0.27</td>
</tr>
<tr>
<td>βR246K</td>
<td>–</td>
<td>50</td>
<td>0.27</td>
</tr>
<tr>
<td>βR246A</td>
<td>–</td>
<td>50</td>
<td>0.27</td>
</tr>
</tbody>
</table>

* Wild-type, pBWW13.4/DK5; Null, pUC118/DK5. All three βArg-246 mutants were expressed with the βY331W mutation also present.

**Fluorescence Titrations**—The method utilizes the quench of fluorescence of introduced residue βTrp-331 that occurs upon nucleotide binding to catalytic sites (34). Titration with MgADP and MgATP was carried out in 50 mM TrisSO4, pH 8.0, 2.5 mM MgSO4 at room temperature. NaADP and NaATP were added incrementally. Excitation was at 285 nm, and emission was at 360 nm in a SPEX Fluorolog 2 spectrofluorometer. Final enzyme concentration in the cuvette was 150–200 nm, and enzyme was incubated for 10 min at room temperature before titration was begun. Background signals due to buffer were subtracted, and volume and inner filter effects were corrected by carrying out parallel titrations with wild-type F1. Calculation of nucleotide binding parameters was accomplished by fitting theoretical curves to the measured data points assuming models with three types of binding sites (34, 35).

**Inhibition of ATPase Activity**—For fluoralanilumine, fluoroscadium, fluoroberyllate, and vanadate inhibition, F1 was 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 0.2–1.0 mg/ml in presence of AlCl3, ScCl3, or BaSO4, added at varied concentrations (see *Results*). 100-μl 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. NaF was absent when vanadate was the inhibitor, and sodium orthovanadate was pretreated as described (36). For NBD-CI inhibition of purified F1, NBD-CI was prepared as a stock solution in dimethyl sulfoxide and protected from light. Enzyme (0.2–0.4 mg/ml) was reacted with NBD-CI for 60 min in the dark, at room temperature. In 150 mM TrisSO4, pH 8.0, 2.5 mM MgSO4, and then 100-μl aliquots were transferred to 1 ml of ATPase assay buffer to determine activity. For NBD-CI inhibition of F1, F1 in membranes, protein concentration was 0.7–1.0 mg/ml in 50-μl aliquots; the other conditions were the same. Where protection from NBD-CI inhibition by ADP or P1 was determined, F1 and membranes were preincubated for 60 min with protecting agent before addition of NBD-CI. MgSO4 was present, equimolar with ADP or P1. Control samples containing the ligand without added NBD-CI were included. Neither P1 (up to 50 mM) nor MgADP (up to 10 mM) had any inhibitory effect alone. For measurement of azide inhibition, sodium azide at varied concentrations was added directly to the ATPase assay buffer, to which F1 was added to start the reaction.

**RESULTS**

Properties of βR246Q, βR246K, and βR246A Mutants of E. coli ATP Synthase—Residue βArg-246 was changed to Glh, Lys, or Ala by oligonucleotide-directed mutagenesis, and then the mutant ATP synthase complex was expressed from a plasmid in a strain of E. coli (DK5) containing a deletion of the ATP synthase genes. It should be noted that the additional mutation βY331W was also present in all three cases, to allow monitoring of nucleotide binding affinity and stoichiometry in purified F1 by fluorescence titration. Effects of the βArg-246 mutations on oxidative phosphorylation in vivo were assayed by growth on succinate-containing plates and in limiting glucose medium. All three mutations prevented growth on succinate. Growth yields on limiting glucose were reduced close to the ATP synthase null control (Table I). Therefore, all three mutations seriously impaired ATP synthesis in vivo. The βY331W mutation alone has little effect on growth.

F1 was purified from each mutant, and yields were similar to that with wild-type enzyme. On SDS gels, the enzymes appeared pure and showed the same subunit composition as in wild type. Analysis of Trp content by measurement of fluorescence in 6 M guanidine hydrochloride (λem = 295 nm, λex = 360 nm) confirmed that the puruity of the mutant enzymes was the same as wild type, with the expected 12 Trp/mol. Specific ATPase activities for the mutant enzymes are given in Table I. The ATPase activity was around 1% of wild type in all three mutant enzymes. Similar activity had been seen earlier in βR246H and βR246C enzymes (21, 22). Retention of the positive charge in the βR246K mutant did not therefore affect the outcome of mutagenesis, at least as far as ATPase activity in vitro or ATP synthesis in vivo were concerned.

Ability of the Mutant F1, to Bind MgATP and MgADP—Fluorescence of introduced residue βTrp-331 was used to measure MgATP binding characteristics (8, 34). Titrations of the purified mutant F1 enzymes with the nucleotides were carried out (data not shown), and Kd values for binding at each of the three catalytic sites, calculated as described under "Experimental Procedures," are listed in Table II. The mutations caused reduction of affinity for MgATP, most noticeably at site two (the site of "medium" affinity) and at site one (highest affinity) with little effect at site three (lowest affinity). Most interesting, the mutation that might have been predicted to have the smallest effect, namely βR246K, in fact had the largest effect. With MgADP there was again loss of affinity at sites one and two, with lesser effects at site three, and the βR246K mutant had the largest effect. It appears, however, that the large effects of the mutations on ATP synthesis and ATPase activity are not commensurately reflected in impairment of substrate ADP or ATP binding affinities. At saturation (1 mM of MgADP or MgATP) all three mutant enzymes would contain all three sites filled, as in wild type.

**Inhibition of ATPase Activity of F1 by Phosphate Analogs Fluoralanilumine, Fluoroscadium, Orthovanadate, and Fluoroberyllate—Wild-type or βY331W E. coli F1 ATPase is strongly inhibited by AlCl3 in combination with NaF, ADP, and Mg2+ (37, 38), and experiments showed that MgADP-fluoroalanilumine formed a tenaciously bound inhibitory complex with F1 that mimicked a transition state (38–40). X-ray crystallography subsequently deduced the structure of mitochondrial F1 with both ADP-AlF4 and ADP-AlF6 species bound (19, 20). Fig. 2A shows that the mutations βR246Q, βR246K, or βR246A all rendered E. coli F1 insensitive to ADP-fluoroalanilumine inhibition. Since the concentration of MgADP in these experiments (1 mM) was easily sufficient to saturate the enzyme catalytic sites (Table II), our conclusion is that substitution of βArg-246 greatly weakens interaction with the fluoroalanilumine moiety. Notably, even the Lys mutant was unable to substitute for Arg.

MgADP-fluoroalanilumine complex was also shown to inhibit wild-type and βY331W F1 ATPase activity potently (41) by mimicking a transition state. Fig. 2B shows that the mutants βR246Q, βR246K, and βR246A were all completely immune to ADP-fluoroalanilumine inhibition, again suggesting that βArg-
246 in wild type is normally important in liganding the fluoroberyllate moiety and bringing about inhibition.

MgADP-vanadate complex is an inhibitory transition state analog that has been used to study a variety of ATPase enzymes. Because the vanadate moiety is pentacovalent in the inhibited enzyme complex (e.g. Ref. 42), ADP-vanadate is thought to provide a close mimic of a true transition state of a natural transphosphorylation reaction (43). Unfortunately, both wild-type and mutant E. coli F₁ proved to be resistant to inhibition by vanadate (up to 2.4 mM) in combination with 1 mM MgADP (data not shown).

The MgADP-fluoroberyllate complex is different from the preceding inhibitors in that the bound complex mimics a ground state with ATP bound (e.g. Ref. 44). MgADP-fluoroberyllate is a potent inhibitor of wild-type E. coli F₁ as shown in Fig. 3. Here a varied pattern of inhibition was seen with the mutant enzymes. βR246K was inhibited to the same extent as wild type (~90%); βR246A was inhibited by ~45% and βR246Q by ~30%. Thus, substitution of Arg impaired liganding to the fluoroberyllate moiety in Ala and Gln mutants but not with Lys.

To summarize, the results show that residue βArg-246 is critical for liganding the phosphate moiety of ATP in the transition state and that it also plays a role in binding the γ-P in the MgADP-fluoroberyllate complex. Lys can substitute in the latter situation but not in the former.

Inhibition of ATPase Activity of F₁ by Azide—Azide is a potent inhibitor of F₁-ATPases in general, although its mode of inhibition remains controversial (45). It has not yet been located in X-ray structures of F₁. One school of thought envisages that azide might form a tightly bound MgADP-N₅ complex at catalytic site(s) (46). However, data in Ref. 45 led to the conclusion that azide blocks conformational signal transmission between catalytic sites. Fig. 4 shows that the mutant βR246Q, βR246K, and βR246A enzymes were all remarkably resistant to inhibition by sodium azide (note that βY331W F₁ is strongly inhibited by azide, see Ref. 45).

Inhibition of ATPase Activity of Purified F₁ and F₁F₀ in Membranes by NBD-Cl in βArg-246 Mutants—NBD-Cl is a potent inhibitor of F₁-ATPase activity that covalently reacts at a stoichiometry of 1 mol/mol F₁, specifically with residue βTyr-297, situated at the end of the P₁ binding pocket and very close to βArg-246 (Fig. 1, A–C). Fig. 1D shows X-ray structure analysis of the empty catalytic site (βE) containing the covalent NBD-O-tyrosyl adduct (47). Our interest in NBD-Cl inhibition was piqued not only because residue βTyr-297 is close to βArg-246 and to the P₁ moiety in catalytic sites but also because Perez et al. (48) had reported that F₁ protects against NBD-Cl inhibition of F₁F₀-ATPase in mitochondrial membrane preparations, potentially providing a tool to assess the role of βArg-246 in P₁ binding.
Initial experiments revealed an interesting difference between mutants and wild type, which was that the former showed only \( \frac{1}{2} \) inactivation by NBD-Cl both in purified F1 (Fig. 5, A–C) or in membranes (Fig. 5, D–F), whereas wild type was almost totally inactivated (we confirmed that \( \beta Y331W \) alone behaved the same as wild type). With the mutant enzymes, approximately the same degree of residual ATPase activity remained in purified F1 as in membranes. It may be noted that NBD-Cl was extremely effective in wild type, with residual ATPase activity amounting to only 0.02 \( \frac{\text{mol/min}}{\text{mg}} \) of purified F1 protein at the higher concentrations of NBD-Cl, whereas residual activities in the mutant purified F1 enzymes were around 0.10–0.12 \( \frac{\text{mol/min}}{\text{mg}} \). Inhibition with NBD-Cl was independent of the presence of \( \text{MgSO}_4 \) in the reaction buffer. Maximal inhibition was reached in 1 h at room temperature with 150 \( \mu \text{M} \) NBD-Cl in the mutant purified F1 (Fig. 5, A–C). If at the end of this period, an additional pulse of NBD-Cl equivalent to extra 200 \( \mu \text{M} \) was added and incubation was continued for an additional hour, little additional inhibition occurred (Fig. 6A). Thus the reason for incomplete inhibition in the mutant enzymes in Fig. 5 was that the fully reacted enzymes retained significant residual activity, whereas in wild type residual activity was negligible. It is notable that all three mutant enzymes behaved similarly. Therefore, it appears that mutation of \( \beta \text{Arg-246} \) affected both reactivity of the proximal residue \( \beta \text{Tyr-297} \) with NBD-Cl and the level of residual ATPase in the fully reacted enzyme. Incubation with 4 mM DTT after maximal inhibition had been achieved restored full activity in all cases (Fig. 6B), establishing that the reaction was specifically with residue \( \beta \text{Tyr-297} \) in the mutant enzymes (49, 50).

**Inhibition of ATPase Activity by NBD-Cl in Membranes and Purified F1; Protection by MgADP and Pi**

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![Image](https://via.placeholder.com/150)

**Fig. 5. Inhibition of purified F1 and membrane-bound F1F0 by NBD-Cl.** Enzyme was preincubated for 60 min at 23 °C with the indicated concentration of NBD-Cl, then aliquots were added to 1 ml of assay buffer, and ATPase activity was determined. For details see “Experimental Procedures.” ○, wild type; •, \( \beta R246Q/\beta Y331W \); ■, \( \beta R246K/\beta Y331W \); ▲, \( \beta R246A/\beta Y331W \). A–C, purified F1; D–F, membrane-bound enzyme.

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FIG. 6. Results of an extra pulse of NBD-Cl in mutant enzymes and reversal of NBD-Cl inhibition by DTT. A, purified F1 was inhibited by reaction with 150 μM NBD-Cl for 60 min under conditions as described in Fig. 5. Then a further pulse of NBD-Cl, equivalent to 200 μM, was added and incubation continued a further 60 min before assay. B, purified F1 was incubated with or without 150 μM NBD-Cl for 60 min as in Fig. 5. Degree of inhibition was assayed. In parallel samples, DTT (4 mM) was then added, and incubation continued for a further 60 min before assay. In each group of histograms, the order is, from left to right, wild type, βR246Q, βR246R, and βR246A.

FIG. 7. MgADP protection of ATPase activity in wild type and mutant purified F1 and membranes from inactivation by NBD-Cl. Purified F1, or membranes were preincubated with varying concentrations of MgADP for 60 min at 23 °C, and then NBD-Cl (100 μM) was added and incubation continued for 60 min at the end of which ATPase activity was assayed. For further details see “Experimental Procedures.” C, wild type; ○, βR246Q/βY331W; ■, βR246K/βY331W; ▲, βR246A/βY331W. A–C, purified F1; D–F, membrane-bound enzyme.
Fig. 8. Protection by P_i of wild-type ATPase activity in membranes and purified F_1 from inactivation by NBD-Cl. Membranes or purified F_1 were preincubated with P_i at 2.5 or 10 mM concentration as shown for 60 min at 23 °C. Then NBD-Cl (100 μM) was added, and aliquots were withdrawn for assay at the time intervals as shown. ATPase activity remaining is plotted against time of incubation with NBD-Cl. A, wild-type membranes. B, wild-type purified F_1. ○, no P_i added; □, 2.5 mM P_i; △, 10 mM P_i.

Fig. 9. Protection by P_i of mutant ATPase activity in membranes from inactivation by NBD-Cl. The procedure was as in Fig. 8. Solid symbols have no P_i added. ●, βR246Q/Y331W; ■, βR246K/Y331W; ▲, βR246A/Y331W. Open symbols have 10 mM P_i added. ○, βR246Q/ Y331W; □, βR246K/Y331W; △, βR246A/Y331W. The same results were seen with 2.5 mM P_i.

Perez et al. (48) found that Pi protected F_1F_0-ATPase activity in mitochondrial membranes from inhibition by NBD-Cl. In initial experiments here with wild-type enzyme in E. coli membranes, or with purified F_1, we found that if Pi (up to 10 mM) was preincubated with enzyme under conditions used to study MgADP protection as in Fig. 7 (i.e. all samples reacted 60 min with 100 μM NBD-Cl), then the maximum degree of protection seen with Pi in wild type was around 25%, and no protection was seen in the mutants. Perez et al. (48), however, used a different experimental approach in which inactivation by NBD-Cl was monitored as a function of time in the absence or presence of Pi. When we used this approach on wild-type E. coli membranes or purified F_1, protection by Pi was evident (Fig. 8). Moreover, no protection was seen with any of the mutants when membrane enzyme was tested (Fig. 9). It was also found that Pi showed no protection in purified mutant F_1 (data not shown).

**DISCUSSION**

The goal of this work was to examine the functional role(s) of residue βArg-246 of E. coli ATP synthase. This residue is located in the phosphate-binding pocket of the catalytic sites (Fig. 1). Pi binding and release are important steps in ATP synthase mechanism, likely coupled to rotational movement of subunits, and from its location βArg-246 could be directly involved. However there is little previous experimental evidence available on the role of this residue. Here we mutated the Arg side chain to Gln (removes charge, preserves bulk), Lys (preserves positive charge), and Ala (removes side chain and charge).

All three mutations had the same effect on function, namely growth by oxidative phosphorylation was severely impeded, and ATPase activity of purified F_1 was ~1% of wild type. Previous work on βR246H and βR246C mutants had given the same results. The first conclusion therefore is that the Arg side chain at this location is critical for function. MgATP and MgADP binding parameters were determined by fluorescence titration. The mutants showed lower affinity for MgATP at the first two catalytic sites, and most interesting, the βR246K mutation produced the largest effect, despite retaining the positive charge. The Ala mutant was least affected. The same pattern was seen for MgADP binding, where deviations from wild type were smaller. From these data it is apparent that the positive charge on βArg-246 is not critical for nucleotide substrate or product binding. Whereas the mutations did produce lessening of MgATP binding affinity, to differing extents, this was not well correlated with impairment of ATPase.

Fluoroaluminate and fluoroscandium in combination with ADP and Mg^{2+} ions inhibit wild-type E. coli F_1 potently and have been shown to increase greatly the affinity for ADP binding at two of the three catalytic sites (38, 41). Both are believed to mimic the chemical transition state, and indeed transition
state-like structures involving the bound MgADP-AlF₄⁻ complex were seen in two catalytic sites by x-ray crystallography (19). Here we found that inhibition by these compounds was abrogated by the βArg-246 mutations, showing that the Arg side chain is critical for binding the analogs. Apparently the transition state that forms in the mutants is different from normal in that coordination of the γ-P is altered. This is consistent with a previously stated conclusion (51) that “mutant βR246C seemed to have a different multisite reaction pathway.” Thus βArg-246 is critical for formation of wild-type transition state and the rapid catalysis that ensues.

Fluoroberyllate in combination with MgADP is usually regarded as a ground state analog of the substrate ATP in the transition state and the rapid catalysis that ensues. Studies that NBD-Cl reacts only in the empty ("ground") state, catalytic intermediate, or dead-end complex is unclear. An x-ray structure of MgADP-BeFₓ trapped in F₁ catalytic sites has not yet been published. Further interpretation of our results will have to await structural analysis.

All three mutants rendered F₁ insensitive to azide inhibition. In mitochondrial and Bacillus PS3 enzymes, azide is thought to inhibit by trapping MgADP at catalytic sites, possibly by acting as a Pᵢ analog in a tightly bound MgADP-Pᵢγ complex (46). However, in E. coli F₁ this did not seem to be the case (45), rather it was suggested that azide interfered with conformational signal transmission between catalytic sites required for rapid catalysis. It had been suggested previously (51) that residue βArg-246 is responsible for such conformational signaling. If signaling is already defunct in the mutant enzymes, with resultant low ATPase, then further inhibition by azide might not be expected. Thus, the data do not necessarily support a direct role for βArg-246 in Pᵢ binding. However, they do reaffirm that the residual ATPase activity seen in the mutant enzymes is going via a different catalytic pathway to normal.

NBD-Cl inhibited the ATPase activity of the mutant enzymes to only ~50%, at full reaction, as compared with essentially 100% in wild type, providing further evidence that the ATPase of the mutants is different in character from wild type. NBD-Cl still reacted with βTyr-297 in the mutants as in wild type. Somewhat higher concentrations of NBD-Cl were required for inhibition in the mutants. As noted in Fig. 1, residue βArg-246 is close to βTyr-297 in wild-type catalytic sites. Orris et al. (47) have concluded from their x-ray crystallography studies that NBD-Cl reacts only in the empty (βE) catalytic site of wild-type enzyme. Our data support the conclusion that the rate of inactivation was linearly dependent on NBD-Cl concentration and only very high concentrations of MgADP gave protection from reaction. We presume that the high concentrations of MgADP are needed in order to keep the βE site inaccessible to NBD-Cl in the time average. Because the same very high concentrations of MgADP were required to protect the mutant enzymes from inhibition, the same conclusion applies to the mutants.

The protection that Pᵢ afforded against NBD-Cl inhibition in wild-type membranes and purified F₁ is of great interest, because it shows that the presence of Pᵢ in the βE site, probably in a position similar to that of SO₄⁻ in the "βADP + Pᵢ" site (Fig. 1D), impedes accessibility and/or reaction of NBD-Cl with βTyr-297. Perez et al. (48) favored the interpretation that protection was due to Pᵢ binding in catalytic sites, but they were not yet in possession of structural information that now solidifies this conclusion and also makes it clear which particular catalytic site conformation is involved. Moreover, no protection by Pᵢ was seen here in any of the mutants, providing evidence that Pᵢ binding was to the βArg-246 side chain in the βE site.

Earlier work from our laboratory (7) reported that in aqueous buffer at pH 6.5–7.5 maximal stoichiometry of [³²P]PPᵢ binding to wild-type E. coli F₁ measured using the centrifuge column technique (54) was 0.125 mol/mol after incubation with 1 mM Pᵢ. It should be noted that the radioactive Pᵢ was first purified to remove PPᵢ and higher polyphosphates, as described previously (55, 56), and that the precaution reduced the amount of Pᵢ₃-bound radioactivity considerably. (Subsequent experiments have shown that PPᵢ (10) and PPPᵢ (18) bind with KD of 20 and 50 μM to the noncatalytic sites of E. coli F₁.) Penczek et al. (54) showed that under the conditions used for centrifuge column elution, the transit time for protein is around 30 s, so that for ~90% retention of bound ligand a dissociation rate ≤0.006 s⁻¹ would be required. As was pointed out in Ref. 7, Pᵢ bound in the highest affinity catalytic site should be retained in centrifuge column experiments, as determined from unisite kinetics of Pᵢ release. It is, however, quite feasible that Pᵢ bound in the βE site would have a dissociation constant sufficiently fast that it would not be retained upon centrifuge column elution. Consistent with this, Perez et al. (48) calculated a KD of 0.2 mM from kinetic experiments involving inactivation of mitochondrial membrane ATPase by NBD-Cl at varied Pᵢ concentration. Our experimental data with E. coli enzyme (Fig. 8) are similar.

We also found earlier that binding of Mg-AMPPNP or ATP (no Mg²⁺) to the three catalytic sites of E. coli F₁ was not competed by 5 mM Pᵢ (8, 9), from which we concluded that the KD of [³²P]PPᵢ at all three of the catalytic sites was >10 mM (10). Results discussed above indicate that the βE site does bind Pᵢ, with KD in the mM range. It is possible that, in the absence of rotation, a βE site with Pᵢ bound can partly close and still accommodate Pᵢ along with Mg-AMPPNP or ATP, so that competition between these ligands is not evident. Presumably during rotational catalysis induced by MgATP, any Pᵢ bound along with MgATP in βE is expelled as full closing of the site occurs, and p₁ in the medium cannot appreciably bind. This would be consistent with the well-established lack of inhibition of ATPase by Pᵢ (e.g., Ref. 57 found no inhibition at 50 mM Pᵢ). In contrast, in proton gradient-driven rotation, Pᵢ bound to βE must be retained as the site closes. How this is achieved is not yet established; however, the work presented here clearly shows that the βArg-246 side chain is an important component in binding Pᵢ and in forming the transition state.

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REFERENCES
14. Fischer, S., Etzold, C., Turina, P., Deckers-Hebestreit, G., Allendorf, K., and...