Modulation of Charge in the Phosphate Binding Site of Escherichia coli ATP Synthase*

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This paper presents a study of the role of positive charge in the P1 binding site of Escherichia coli ATP synthase, the enzyme responsible for ATP-driven proton extrusion and ATP synthesis by oxidative phosphorylation. Arginine residues are known to occur with high propensity in P1 binding sites of proteins generally and in the P1 binding site of the βE catalytic site of ATP synthase specifically. Removal of natural βArg-246 (β246A mutant) abrogates P1 binding; restoration of P1 binding was achieved by mutagenesis of either residue βAsn-243 or αPhe-291 to Arg. Both residues are located in the P1 binding site close to βArg-246 in x-ray structures. Insertion of one extra Arg at β-243 or α-291 in presence of βArg-246 retained P1 binding, but insertion of two extra Arg, at both positions simultaneously, abrogated it. Transition state stabilization was measured using phosphate analogs fluoroaluminate and fluorescent roscamund. Removal of βArg-246 in β246A caused almost complete loss of transition state stabilization, but partial rescue was achieved in βN243R/β246A and αF291R/β246A. βArg-243 or αArg-291 in presence of βArg-246 was less effective; the combination of αF291R/β246A with natural βArg-246 was just as detrimental as β246A. The data demonstrate that electrostatic interaction is an important component of initial P1 binding in catalytic site βE and later at the transition state complex. However, since none of the mutants showed significant function in growth tests, ATP-driven proton pumping, or ATPase activity assays, it is apparent that specific stereoechemical interactions of catalytic site Arg residues are paramount.

ATP synthase is the terminal enzyme of oxidative phosphorylation and photophosphorylation, which synthesizes ATP from ADP and phosphate (P1). The energy for ATP synthesis comes from transmembrane movement of protons down an electrochemical gradient, generated by substrate oxidation or by light capture. Initially, as the protons move through the interface between α and c subunits in the membrane-bound F0-sector of the enzyme, the realized energy is transduced into mechanical rotation of a group of subunits (γεc10–14), which comprise the “rotor”. A helical coiled coil domain of γ projects into the central space of the αββ3 hexagon, in the membrane-extrinsic F1-sector. αββ3 hexagon contains three catalytic sites at α/β interfaces. In a manner that is not yet understood, rotation of γ vis-à-vis the three αβ subunit pairs brings about ATP synthesis at the three catalytic sites using a sequential reaction scheme (1). “Stator” subunits b2 and δ are present to prevent co-rotation of αββ3 with the rotor. Detailed reviews of ATP synthase mechanism may be found in Refs. 2–5.

Binding of P1 is an important step of the ATP synthase mechanism that has been extensively studied by biochemical approaches and may be directly coupled to rotation of subunits (3, 6–11). Recent studies of the rotational mechanism have begun to illuminate which steps in the enzymatic pathway of ATP synthesis and hydrolysis are likely coupled to the two substeps (80° and 40°) of subunit rotation and which steps occur in the intervening stationary dwells (12–15). While it has not yet been possible to directly correlate the step of P1 binding/release with a specific mechanical event or an intervening dwell, it seems likely that this will soon be achieved. Thus we can foresee that it may be possible in the near future to correlate molecular features of P1 binding, derived from mutational and biochemical studies, with mechanical function in this nanomotor system.

Studies of molecular aspects of P1 binding in ATP synthase have been held back by lack of a suitable system to which both mutagenesis and a P1 binding assay were applicable. Penevsky (16, 17) reported that P1 binding to mitochondrial ATP synthase F1 could be assayed using the centrifuge column procedure with an estimated Kd(P1) of 30 μM. However Al-Shawi and Senior (8) found that in Escherichia coli F1, no P1 binding was detectable by this procedure. Further work by Weber and colleagues (18–20) was carried out to determine whether P1 binding could be assayed in E. coli F1 using competition assays with MgAMPPNP or ATP, but these attempts also proved negative. 7-Chloro-4-nitrobenzo-2-oxa-1,3-diazole (NBD-CI)1 is a potent inhibitor of ATPase activity that covalently reacts at stoichiometry of 1 mol/mol ATP synthase, specifically with residue βTyr-297,2 situated at the end of the P1 binding pocket (21–23). Following the terminology of Walker, Leslie, and colleagues (23), the three catalytic sites are conventionally referred to as βE, βDP, and βTP. NBD-CI was found to react in the βE (empty) site. Perez et al. (24) reported that P1 protects against NBD-CI inhibition of ATPase activity of ATP synthase in mitochondrial membrane preparations, potentially providing a tool to assay P1 binding in βE catalytic site. From their work, a Kd(P1) of 0.2 mM was calculated. In recent work we confirmed that this assay was applicable, both with membrane-bound enzyme and with purified F1 from E. coli (11). Concentration dependence of P1 protection against NBD-CI inactivation in E. coli enzyme was similar to that found by Perez et al. (24) in

1 The abbreviations used are: NBD-CI, 7-chloro-4-nitrobenzo-2-oxa-1,3-diazole; DTT, dithiothreitol; TES, 2-(2-hydroxy-1,1-bis(hydroxymethyl)ethyl)aminioethanesulfonic acid.
2 E. coli residue numbers are used throughout.

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mitochondrial enzyme. Studies of NBD-Cl inactivation kinetics and of MgADP protection characteristics confirmed that reaction occurred in the βE site in E. coli enzyme (11). Subsequently using mutagenesis we found this assay to be successful in assessing the functional roles of various catalytic site residues in Pᵢ binding (11, 25, 26). X-ray crystal structures of catalytic sites containing the Pᵢ analogs AlF₃ (27) and SO₄²⁻ (28) were valuable in suggesting residues within the Pᵢ binding pocket that were suitable targets for mutagenesis. Finally it may be noted that Penevsky (29) has recently confirmed, using purified [³²P]Pᵢ, that Pᵢ binding to E. coli F₁ is not detectable by the centrifuge column procedure but that a pressure ultrafiltration method did detect Pᵢ binding, with a Kᵦ₆ (Pᵢ) in the range of 0.1 mM, consistent with data obtained from the NBD-Cl inactivation assay. It is apparent that Pᵢ dissociates more rapidly from E. coli F₁ than it does from mitochondrial F₁, unfortunately rendering the convenient centrifuge assay inapplicable with the E. coli enzyme.

In proteins, arginine residues show the highest propensity for occurrence and functional interaction at Pᵢ binding sites (30). Our earlier work established that natural Arg residues at positions α-376, β-182, and β-246 were important for Pᵢ binding in the βE catalytic site of ATP synthase, with the latter playing a key role (11, 26). Mutagenesis of βArg-246 to Ala, Gln, or Lys abolished Pᵢ binding (11). Residue βAsn-243, although totally conserved and located very close to bound Pᵢ, was found to be not directly involved in interacting with Pᵢ. Rather it was found to be necessary for correct organization of the transition state complex (25). However, if Asp was introduced at this position it prevented Pᵢ binding, presumably because it nullified the positive charge of the neighboring βArg-246 (25). Therefore balance of charge in the Pᵢ binding pocket also appeared important.

After binding, Pᵢ must be condensed with MgADP via a chemical transition state, for which a molecular mechanism has been proposed in (3). The transition state analog MgADP-AlF₃ trapped in catalytic sites has been visualized by x-ray crystallography (28), and it is clear that the fluoride-alumininate group occupies the position of phosphate in the transition state complex. Contribution of different residues to stabilization of the transition state complex can be compared by assay of inhibition of ATPase activity by MgADP-fluoroaluminate (or MgADP-fluoroorcinol) in mutant and wild-type enzymes (11, 25). By comparing effects on Pᵢ binding and transition state stabilization one can further infer roles of each potential Pᵢ residue at early and later steps of the catalytic pathway.

In this paper we modulated charge within the Pᵢ binding site by introduction of extra Arg at residues β-243 and α-291, both in the presence of the natural βArg-246 and in its absence (βR246A mutant). We also combined βArg-243 and αArg-291 with the natural βArg-246 to test effects of excess positive charge. Pᵢ binding and transition state stabilization were assessed in each of the new mutants.

MATERIALS AND METHODS

Preparation of E. coli Membranes; Measurement of Growth Yield in Limiting Glucose Medium; Assay of ATPase Activity of Membranes; Measurement of Proton Pumping in Membrane Vesicles; SDS-gel Electrophoresis; Immuno blotting—E. coli membranes were prepared as described previously (31). It should be noted that this procedure involves three washes of the initial membrane pellets, once in buffer containing 50 mM TES, pH 7.0, 15% glycerol, 40 mM 6-aminohexanoic acid, 5 mM p-aminobenzenemidazime, then twice in buffer containing 50 mM TES, pH 7.0, 15% glycerol, 40 mM 6-aminohexanoic acid, 5 mM p-aminobenzenemidazime, 0.5 mM DTT, 0.5 mM EDTA. Prior to the experiments, membranes were washed twice more by resuspension and ultracentrifugation in 50 mM Tris/Sac₃, pH 8.0, 2.5 mM MgCl₂. Growth yield in limiting glucose was measured as described previously (32). ATPase activity was measured in 1 ml of assay buffer containing 10 mM NaATP, 4 mM MgCl₂, 50 mM Tris/Sac₃, pH 8.5 at 37 °C. Reactions were started by addition of membranes and stopped by addition of SDS to 3.3% final concentration. Pᵢ released was assayed as described previously (33). For wild-type membranes (5–10 μg of protein), reaction times were 2–10 min. For mutant membranes (20–100 μg of protein), reaction times were 30–120 min. All reactions were shown to be linear with time and protein concentration. ATP-driven proton pumping was measured by following the quench of acidine orange fluorescence as described previously (34). SDG-gel electrophoresis on 10% acrylamide gels was as described previously (35). Immunoblotting with rabbit polyclonal anti-F₆-α and anti-F₇-β antibodies was as described previously (36). Densitometry of immunobots was performed using software from Scion Corp. (Scion Image Release Beta 4.02, www.scioncorp.com/).

Construction of Mutant Strains of E. coli—Mutagenesis was by the method of Vandeyar et al. (38). For βR243R/βR246A and βN243R mutants, the template for oligonucleotide-directed mutagenesis was M13mp18 containing the HindIII-Xbal fragment from pSN6. pSN6 is a plasmid containing the βY331W mutation also present, which does not significantly affect growth. Data are means of four to six experiments each.

TABLE I

| Mutation | Growth on succinate | Growth yield in limiting glucose |
|----------|---------------------|----------------------------------|
| Null     | +++++               | 100                              |
| βR246A   | +                   | 46                               |
| βN243R   | +                   | 55                               |
| βN243R/βR246A | +       | 57                               |
| αF291R   | +                   | 59                               |
| αF291R/βR246A | +      | 57                               |
| αF291R/βN243R | +     | 56                               |

Wild-type, pBWU13.4/DK8; null, pUC118/DK8. All mutants were expressed with the βY331W mutation also present, which does not significantly affect growth. Data are means of four to six experiments each.

a Growth on succinate plates after 3 days estimated by eye. +++++, heavy growth; +, no growth; +, light growth.
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Table II

| Mutation           | ATPase activity$^a$ | Proton pumping activity$^a$ |
|--------------------|---------------------|-----------------------------|
| Wild-type$^c$      | 14.7 ± 2.21 (24)    | 84                          |
| Null mutant (1)$^e$| 0.0013 ± 0.0008 (16)$^b$ | 0                           |
| Null mutant (2)$^e$| 0.0016 ± 0.0004 (16)$^b$ | 0                           |
| $\beta$R246A       | 0.050 ± 0.0059 (16) | 0                           |
| $\beta$N243R       | 0.023 ± 0.0037 (20) | 0                           |
| $\beta$N243R/$\beta$R246A | 0.016 ± 0.0046 (24) | 0                           |
| $\alpha$F291R      | 0.033 ± 0.0051 (23) | 0                           |
| $\alpha$F291R/$\beta$R246A | 0.52 ± 0.11 (24) | 3                           |
| $\alpha$F291R/$\beta$N243R | 0.028 ± 0.0033 (20) | 0                           |

$^a$ Measured at 37°C and expressed as μmol ATP hydrolyzed/min/mg of membrane protein. Data are given as mean ± standard deviation with number of individual experimental points in parentheses. Each individual experimental point is itself the mean of duplicate assay tubes. Data are derived from three ($\beta$R246A, $\beta$N243R/$\beta$R246A) or two (all others) separate membrane preparations. Results from separate membrane preparations were in excellent agreement.

$^b$ Measured using acridine orange and expressed as per cent quench of acridine orange fluorescence in membrane vesicles upon addition of 1 mM MgATP.

$^c$ Wild type, pBWU13.4/DK8; null mutants, 1) pUC118/DK8 and 2) DK8.

$^d$ The very low ATPase activity in the null mutants is attributable to the fact that all membrane preparations were washed several times before assays (see "Materials and Methods").

ml of ATPase assay buffer to determine ATPase activity. Where protection from NBD-Cl inhibition by ADP or $P_i$ was determined, membranes were preincubated 60 min with protecting agent at room temperature before addition of NBD-Cl. MgSO$_4$ was present, equimolar with ADP or $P_i$. Control samples containing the ligand without added NBD-Cl were included. Neither Pi (up to 50 mM) nor MgADP (up to 10 mM) had any inhibitory effect alone. Where reversal of NBD-Cl inhibition by DTT was measured, membranes were first reacted with NBD-Cl (150 μM) for 1 h at room temperature, then DTT (final = 4 mM) was added and incubation continued for 1 h at room temperature before ATPase assay. Control samples without NBD-Cl and/or DTT were incubated for the same times.

Inhibition of ATPase Activity by Fluoroaluminate or Fluoroscanium—Membranes were incubated for 60 min at room temperature in 50 mM Tris/SO$_4$, 2.5 mM MgSO$_4$, 1 mM NaADP, and 10 mM NaF at a protein concentration of 0.2–1.0 mg/ml in the presence of AlCl$_3$ or ScCl$_3$ added at varied concentration (see "Results"). 50-mM aliquots were then added to 1 ml of ATPase assay buffer and activity measured as above. It was confirmed in control experiments that no inhibition was seen if MgSO$_4$, NaADP, or NaF was omitted.

RESULTS

Growth Properties of New Mutants of E. coli ATP Synthase—A series of mutants was generated to modulate charge in the proximity of residue $\beta$Arg-246, which was shown earlier to be a key residue for binding of $P_i$ into the catalytic sites on the $F_1$-sector of ATP synthase (11). Mutation of $\beta$Arg-246 to Ala abrogates $P_i$ binding (11). We introduced Arg at two residues located close to $\beta$Arg-246, namely $\beta$Asn-243 and $\alpha$Phe-291, to generate the new mutants $\beta$N243R, $\beta$N243R/$\beta$R246A, $\alpha$F291R, and $\alpha$F291R/$\beta$R246A. These mutants are designed to test the

"Materials and Methods," were run on 10% SDS-polyacrylamide gels together with purified wild-type $F_1$ (0.1–0.4 μg) as reference. Protein bands were transferred to nitrocellulose and immunoblotted using anti-$F_\gamma$-α antibody (36). Densitometry was performed as described under "Materials and Methods." A, immunoblot. B, densitometric scans. The same numbering system applies in A and B. Lanes 1–3, purified $F_\gamma$, 0.1, 0.2, and 0.4 μg, respectively. Lanes 4 and 5, membranes from null mutants DK8 (lane 4) and pUC118/DK8 (lane 5). Lane 6, wild-type (pBWU13.4/DK8) membranes. Lanes 7–12, mutant membranes $\beta$R246A (lane 7), $\beta$N243R/$\beta$R246A (lane 8), $\alpha$F291R/$\beta$R246A (lane 9), $\alpha$F291R (lane 10), $\beta$N243R (lane 11), and $\beta$N243R/$\alpha$F291R (lane 12). Area under the curve (between the tick marks shown) for each membrane preparation is reported on the right of each scan, relative to wild type (lane 6), which is arbitrarily set at 100.
effects of introducing one extra Arg close to βArg-246 and to find out whether loss of βArg-246 can be compensated by introduction of another Arg close by. Mutant αF291R/βN243R tests the effect of having Arg at all three locations: αPhe-291, βAsn-243, and the natural βArg-246.

Growth yields on limiting glucose medium and growth on succinate plates are shown in Table I. It was evident that introduction of a new Arg residue at α-291 or β-243 was debilitating either in combination with or in absence of the β246A mutation, although it may be noted that the β246A mutation alone consistently displayed even lower growth. Similar results were seen in αF291R/βN243R. Therefore oxidative phosphorylation is defective in each of the mutants containing Arg at α-291 or β-243 or both.

SDS-gel Electrophoresis and Immunoblotting of Membrane Preparations—Previous work (11) had established that F1 binding by mutant and wild-type ATP synthase can be assayed using either membrane preparations or purified F1. For a series of mutants, as studied here, it was more efficient to use membrane preparations. However, the possibility existed that the mutations may have compromised assembly and/or oligomeric stability, leading to membrane preparations with low ATP synthase content. This could account for the low growth yields in Table I. We therefore performed SDS-gel electrophoresis and immunoblotting experiments.

Coomassie Blue-stained SDS-gels of mutant and wild-type membranes (with purified wild-type F1 as reference) established that all the mutant membrane preparations had bands running at the position of F1-α and F1-β subunits, with similar intensities to the α and β bands seen in wild-type membranes (data not shown). Immunoblotting and densitometry was performed with anti-α subunit and anti-β subunit antibodies (36). Preliminary experiments using purified wild-type F1 revealed that the response was linear in the range 0.1–0.4 μg of protein, and further tests showed that 4 μg of wild-type or mutant membrane preparations gave a response that fell within this range. An immunoblot using anti-F1-α subunit is shown in Fig. 1A. Purified F1 (0.1–0.4 μg) is run in lanes 1–3 for reference. Membranes (4 μg) from null mutant strains DK8 and pUC118/DK8 are run in lanes 4 and 5, respectively, and show no α subunit, as expected. Lane 6 shows wild-type membranes, and lanes 7–12 show the mutant membranes. A densitometric scan of each lane is presented in Fig. 1B, using the same numbering system. Wild-type membranes (lane 6) are set arbitrarily at 100 (area under the curve), and the density in other membrane preparations (null, lanes 4 and 5; mutants, lanes 7–12) are
presented relative to wild type. Three different experiments gave similar results. It is evident that the mutant membranes were similar in ATP synthase content to wild type. Immuno-blotting using anti-F$_1$-H$_9$ antibody (data not shown) confirmed this conclusion.

ATPase Activity and Proton Pumping Activities of Mutant ATP Synthase Enzymes in Membranes—Table II shows the ATPase and proton pumping activities of the mutant ATP synthase enzymes in membranes compared with wild type and with two different null controls. It may be noted that the membrane preparations were washed extensively before assay. Data from the null controls showed that this removed virtually all contaminating ATPase activity. The following conclusions are evident. First, insertion of one or two new Arg residues close to the Pi binding site (F$_2$91R, N$_2$43R, F$_2$91R/N$_2$43R) in otherwise wild-type background (i.e. with R$_{246}$) reduced membrane ATPase activity to a very low level. ATPase activities were far too low to support ATP-driven proton pumping. Second, insertion of N$_{243}$R in presence of Ala$_{246}$ (N$_2$43R/R$_{246}$A) did not restore ATPase activity. Third, insertion of Arg$_{291}$ in presence of Ala$_{246}$ (F$_2$91R/R$_{246}$A) did significantly restore ATPase activity (by 10-fold over R$_{246}$A alone), and in this case there was detectable, although low, ATP-driven proton pumping. It is apparent that the effects seen on ATPase and proton pumping are consistent with growth characteristics described in Table I; in the case of F$_2$91R/R$_{246}$A the partial “rescue” of R$_{246}$A was apparently not substantial enough to translate into significant growth.

Inhibition of ATPase Activity of ATP Synthase in Membranes by NBD-Cl—Fig. 2 shows NBD-Cl inhibition of each of the new mutant ATP synthase enzymes generated in this work, together with wild type and R$_{246}$A mutant for comparison. In each panel the mutant enzyme is represented by filled circles and wild type by open circles. Please note that the 100% value in each case is the uninhibited ATPase rate as shown in Table II; this rate varied widely in wild type versus the different mutants. However percent inhibition is plotted to allow easy comparison of the degree of inhibition by NBD-Cl. Wild type was almost completely inhibited by NBD-Cl at higher concentrations. The data show that each mutant enzyme was inhibited by NBD-Cl but to a lesser final extent than in wild type and with different concentration dependence. In previous work (11, 26) we have noted several instances where mutant ATP synthases were incompletely-inhibited by NBD-Cl. To test whether the residual activity was a real activity of NBD-Cl-inhibited enzyme, for each mutant in Fig. 1 we first incubated for 1 h with 150 mM NBD-Cl, then added a further pulse of NBD-Cl, equivalent to additional 200 mM NBD-Cl, and incubated for a further hour before assaying ATPase activity. In each case additional inhibition of ATPase was small or zero, consistent with Fig. 1 data. Two lines of evidence further supported the idea that ATPase activity in mutant membranes is due to ATP synthase. First, in each case inhibition by NBD-Cl was completely reversed, up to starting activity, by incubation of inhibited enzyme with 4 mM DTT at room temperature. This also occurred in wild type and is known to be due to release of the NBD-adduct from Tyr$_{297}$, the reactive residue (21, 22). Second, reaction with NBD-Cl was prevented by presence of MgADP in the reaction incubation, and in each case dependence on MgADP concentration was the same as in wild type (EC$_{50}$ = 4.5 mM). This protection is referable to loose
MgADP binding in catalytic site \( \beta E \) where NBD-Cl reacts (11, 23).

**Inhibition of ATPase Activity by NBD-Cl in Membranes Containing Mutant ATP Synthase; Protection by Pi**—Fig. 3 demonstrates that Pi protected well against NBD-Cl inhibition of ATPase activity in wild type but not in \( \beta R246A \) mutant, confirming previous work (11). It is seen that mutants \( \beta N243R/\beta R246A \) and \( \alpha F291R/\beta R246A \) both showed clear protection by Pi. It is apparent that insertion of an Arg at position \( \beta-243 \) or \( \alpha-291 \) compensates for the loss of the natural Arg at \( \beta-246 \) in Pi binding. Pi binding was retained in \( \alpha F291R \) and \( \beta N243R \). Therefore introduction of one extra Arg did not interrupt Pi binding. However, introduction of two extra Arg (\( \alpha F291R/\beta N243R \) mutant) prevented Pi binding.

**Inhibition of ATPase Activity by Fluoroaluminate and Fluoroscandium in Membranes Containing Mutant ATP Synthase**—Fig. 4 shows inhibition of ATPase activity by fluoroaluminate in each of the mutants (closed circles) as compared with wild type (open circles). The top left panel shows results obtained for \( \beta R246A \) and wild-type membranes, and it may be noted that the data are very similar to the parallel data reported previously (11) where purified \( \beta \) was used. Wild type was very strongly inhibited (>95%), and \( \beta R246A \) was inhibited by 15% at the highest AlCl3 concentration. Inclusion of “replacement” Arg in \( \beta N243R/\beta R246A \) or \( \alpha F291R/\beta R246A \) mutants increased inhibition markedly, to maximally 42 and 62%, respectively. Inclusion of one additional Arg (\( \beta N243R, \alpha F291R \)) gave maximal inhibition of 94 and 45%, respectively, i.e. less than wild type by far, and less than when in combination with Ala-246 but higher than Ala-246 alone. Inclusion of two additional Arg (\( \alpha F291R/\beta N243R \)) gave 17% inhibition, similar to \( \beta R246A \). An exactly similar pattern was seen when fluoroscandium was the inhibitor (Fig. 5). The maximal inhibition reached with fluoroscandium was: >98% for wild type, 4% for \( \beta R246A \), 28% for \( \beta N243R/\beta R246A \), 62% for \( \alpha F291R/\beta R246A \), 24% for \( \beta N243R \) alone, 49% for \( \alpha F291R \) alone, and zero for \( \alpha F291R/\beta N243R \).

**DISCUSSION**

Pi binding is a primary step in ATP synthesis by ATP synthase, so that understanding the molecular basis of Pi binding is an important goal. Earlier work using the NBD-Cl inactivation assay described in the Introduction has shown that positively charged residues are functionally important for Pi binding in the \( \beta E \) catalytic site of \( E. \) coli ATP synthase (11, 26). X-ray crystallography structures of ATP synthase catalytic sites containing ADP with bound AlF3 or SO42− as phosphate analogs are consistent with these conclusions. To-
Together these studies are supportive of the molecular mechanism for ATP synthesis proposed in (3). Other work (25) indicated that introduction of negative charge in the Pi binding pocket, close to βArg-246, prevented Pi binding. This suggested that modulation of charge in the Pi binding site could be used to illuminate the molecular mechanism of Pi binding. It is established that Arg residues occur particularly commonly in Pi binding sites in proteins (30), therefore varying the number

FIG. 5. Inhibition of membrane ATPase activity from mutant and wild-type ATP synthase enzymes by fluoroscandium. Membranes were preincubated for 60 min at 23 °C with 1 mM MgADP, 10 mM NaF, and the indicated concentration of ScCl₃, then aliquots were added to 1 ml of assay buffer and ATPase activity determined. For details see “Materials and Methods.” ○, wild type; ●, mutant, as indicated in the separate panels. Each data point represents average of duplicate experiments.

FIG. 6. Spatial relationship of residues βAsn-243, βArg-246, and αPhe-291 to AlF₃ and SO₄²⁻ bound in catalytic sites of ATP synthase. Rasmol software was used to generate these figures from the x-ray structures. A, AlF₃ in the βDP catalytic site of AlF₄⁻–inhibited enzyme (27). B, SO₄²⁻ in the βE site of AlF₄⁻–inhibited enzyme (28). E. coli residue numbering is shown, with corresponding bovine mitochondrial residue numbers in parentheses.
of Arg residues in the P_i binding site of ATP synthase seemed a useful approach.

Residue βAsn-243 lies 3.2 Å from βArg-246 in both AlF_3 and SO_4^- containing catalytic sites (nearest atom distances quoted) and close to either AlF_3 or SO_4^- (see Fig. 6). Thus one experimental approach was to introduce the mutation βN243R in wild-type background (with βArg-246) and in presence of the βR246A mutation. Residue αPhe-291, located at the end of the P_i binding pocket across the catalytic α/β interface with its side chain pointing toward the bound P_i analogs, also appeared to be a suitable location at which to introduce a new Arg. It lies at a distance from βArg-246 of 3.2 Å in the AlF_3-containing catalytic site and 7.5 Å in the SO_4^- containing catalytic site (27, 28).\(^3\) We introduced the αF291R mutation in wild-type background and in the presence of the βR246A mutation. Table III shows the actual distances of residues βArg-246, βAsn-243, and αPhe-291 from bound AlF_3 and SO_4^- as determined by x-ray crystallography (27, 28), together with speculative distances (in parentheses) calculated for mutant residues βAla-246, βArg-246 and αArg-291 using the “Deep View Swiss-Pdb Viewer” (described in Table III). It is apparent that the mutations would place extra positive charge relatively close to P_i and that the βAla-246 mutation leaves a relatively large “hole” into which a new Arg might fit. No other suitable location at which to introduce new Arg close to bound P_i was apparent.

SDS-gel electrophoresis and immunoblotting (Fig. 1 and “Results”) showed that the mutant ATP synthase enzymes were present in membrane preparations in amounts that did not deviate strongly from wild type. Growth, ATPase, and ATP-driven proton pumping activities were impaired in all the mutants as compared with wild type (Tables I and II). Introduction of one or two extra positively charged Arg residues in the wild-type background at either β-243 or α-291, or both, was therefore detrimental. Introduction of new Arg at β-243 or α-291 in the βR246A background did not restore function to normal, although a significant compensatory effect on ATPase and ATP-driven proton pumping was seen in the latter case (αF291R/βR246A, Table II).

The βR246A mutant did not show P_i binding but both βN243R and αF291R mutations “rescued” P_i binding in combination with βAla-246 (Fig. 3). Since neither βArg-246 nor αPhe-291 could be expected to assume the same exact stereochemical interactions that βArg-246 achieves, 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. βN243R or αF291R in wild-type background (representing one extra positive charge) did not prevent P_i binding (Fig. 3), but the combination of αF291R/βN243R (two extra charges) abrogated P_i binding. Presumably the local concentration of charge in the latter becomes too disruptive and distorts the P_i binding site.

A similar pattern of effects was seen when transition state stabilization was assessed by assaying inhibition of ATPase activity by the transition state analogs MgADP-fluorooaluminate and MgADP-fluoroscandium. It was shown previously in Ref. 11 that both inhibitors are potent against wild-type ATP synthase but inhibit βR246A mutant only to small extent, indicating that βArg-246 is intimately involved in transition state stabilization. It was found here (Figs. 4 and 5) that mutant residues βArg-243 or αArg-291 partly rescued transition state stabilization when present with βAla-246. Raising the number of positively charged residues to two (βN243R and αF291R mutants in wild-type background) had an adverse effect as reflected by lesser inhibition of ATPase; and raising the number of local positive charges to three reduced transition state stabilization right back to where it was in βR246A. Even in the best cases among the mutants (βN243R/βR246A and αF291R/βR246A) transition state stabilization was incomplete as compared with wild type, providing one explanation for the functional impairment seen in all the mutants.

In summary our results show that in the catalytic site βE of ATP synthase, P_i binding is notably affected by local positive charge. Positive charge in the vicinity of the natural βArg-246 is important; its removal in βR246A mutant can be compensated for partially by introduction of one Arg at either β-243 or α-291. Thus, electrostatic interaction is an important determinant of P_i binding. The presence of two Arg by introduction of either βArg-243 or αArg-291 in presence of βArg-246 does not prevent P_i binding, but the presence of all three Arg abrogates P_i binding. Effects on transition state stabilization followed a parallel pattern. However, restoration of P_i binding in βE catalytic sites by charge compensation is not sufficient by itself to restore full function.

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\(^3\) Introduction of Asp or Glu at α-291 completely prevented P_i binding (Z. Ahmad, unpublished work) indicating proximity of the side chain to bound P_i and βArg-246.
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