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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 P_i 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 P_i binding sites of proteins generally and in the P_i binding site of the βE catalytic site of ATP synthase specifically. Removal of natural βArg-246 (βR246A mutant) abrogates P_i binding; restoration of P_i binding was achieved by mutagenesis of either residue βAsn-243 or αPhe-291 to Arg. Both residues are located in the P_i 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 P_i binding, but insertion of two extra Arg, at both positions simultaneously, abrogated it. Transition state stabilization was measured using phosphate analogs fluoroaluminate and fluoroscandium. Removal of βArg-246 in βR246A caused almost complete loss of transition state stabilization, but partial rescue was achieved in βN243R/βR246A and αF291R/βR246A. βArg-243 or αArg-291 in presence of βArg-246 was less effective; the combination of αF291R/βN243R with natural βArg-246 was just as detrimental as βR246A. The data demonstrate that electrostatic interaction is an important component of initial P_i 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 stereochemical 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 (P_i). 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 *a* and *c* subunits in the membrane-bound F_0-sector of the enzyme, the realized energy is transduced into mechanical rotation of a group of subunits ($\gamma\epsilon c_{10-14}$), which comprise the "rotor". A helical coiled coil domain of γ projects into the central space of the $\alpha_3\beta_3$ hexagon, in the membrane-extrinsic F_1-sector. $\alpha_3\beta_3$ 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 α/β subunit pairs brings about ATP synthesis at the three catalytic sites using a sequential reaction scheme (1). "Stator" subunits b_2 and δ are present to prevent co-rotation of $\alpha_3\beta_3$ with the rotor. Detailed reviews of ATP synthase mechanism may be found in Refs. 2–5.

Binding of P_i 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 enzymic 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 P_i 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 P_i binding, derived from mutational and biochemical studies, with mechanical function in this nanomotor system.

Studies of molecular aspects of P_i binding in ATP synthase have been held back by lack of a suitable system to which both mutagenesis and a P_i binding assay were applicable. Penefsky (16, 17) reported that P_i binding to mitochondrial ATP synthase F_1 could be assayed using the centrifuge column procedure with an estimated $K_d(P_i)$ of 30 μM. However Al-Shawi and Senior (8) found that in *Escherichia coli* F_1, no P_i binding was detectable by this procedure. Further work by Weber and colleagues (18–20) was carried out to determine whether P_i binding could be assayed in *E. coli* F_1 using competition assays with MgAMPPNP or ATP, but these attempts also proved negative. 7-Chloro-4-nitrobenzo-2-oxa-1,3-diazole (NBD-Cl)[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 P_i 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-Cl was found to react in the βE (empty) site. Perez *et al.* (24) reported that P_i protects against NBD-Cl inhibition of ATPase activity of ATP synthase in mitochondrial membrane preparations, potentially providing a tool to assay P_i binding in βE catalytic site. From their work, a $K_d(P_i)$ of 0.2 mM was calculated. In recent work we confirmed that this assay was applicable, both with membrane-bound enzyme and with purified F_1 from *E. coli* (11). Concentration dependence of P_i protection against NBD-Cl inactivation in *E. coli* enzyme was similar to that found by Perez *et al.* (24) in

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[1] The abbreviations used are: 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.

[2] *E. coli* residue numbers are used throughout.

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 P_i binding (11, 25, 26). X-ray crystal structures of catalytic sites containing the P_i analogs AlF_3 (27) and SO_4^{2-} (28) were valuable in suggesting residues within the P_i binding pocket that were suitable targets for mutagenesis. Finally it may be noted that Penefsky (29) has recently confirmed, using purified [^{32}P]P_i, that P_i binding to *E. coli* F_1 is not detectable by the centrifuge column procedure but that a pressure ultrafiltration method did detect P_i binding, with a $K_d(P_i)$ in the range of 0.1 mM, consistent with data obtained from the NBD-Cl inactivation assay. It is apparent that P_i dissociates more rapidly from *E. coli* F_1 than it does from mitochondrial F_1, 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 P_i binding sites (30). Our earlier work established that natural Arg residues at positions α-376, β-182, and β-246 were important for P_i 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 P_i binding (11). Residue βAsn-243, although totally conserved and located very close to bound P_i, was found to be not directly involved in interacting with P_i. 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 P_i binding, presumably because it nullified the positive charge of the neighboring βArg-246 (25). Therefore balance of charge in the P_i binding pocket also appeared important.

After binding, P_i 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-AlF_4^- trapped in catalytic sites has been visualized by x-ray crystallography (28), and it is clear that the fluoroaluminate 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-fluoroscandium) in mutant and wild-type enzymes (11, 25). By comparing effects on P_i binding and transition state stabilization one can further infer roles of each potential P_i residue at early and later steps of the catalytic pathway.

In this paper we modulated charge within the P_i binding site by introduction of extra Arg at residues β-243 and α-291, both in 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. P_i 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; Immnunoblotting—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*-aminobenzamidine, then twice in buffer containing 5 mM TES, pH 7.0, 15% glycerol, 40 mM 6-aminohexanoic acid, 5 mM *p*-aminobenzamidine, 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/SO_4, pH 8.0, 2.5 mM $MgSO_4$. 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 $MgCl_2$, 50 mM Tris/SO_4, pH 8.5 at 37 °C. Reactions were started by addition of membranes and stopped by addition of SDS to 3.3% final concentration. P_i 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 acridine orange fluorescence as described previously (34). SDS-gel electrophoresis on 10% acrylamide gels was as described previously (35). Immunoblotting with rabbit polyclonal anti-F_1-α and anti-F_1-β antibodies was as described previously (36). Densitometry of immunoblots was performed using software from Scion Corp. (Scion Image Release Beta 4.02, www.scioncorp.com/).

TABLE I
Effects of mutations on cell growth

Mutation[a]	Growth on succinate[b]	Growth yield in limiting glucose
		%
Wild-type	++++	100
Null	−	46
βR246A	−	50
βN243R	+	55
βN243R/βR246A	+	57
αF291R	+	59
αF291R/βR246A	+	57
αF291R/βN243R	+	56

[a] 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.

[b] Growth on succinate plates after 3 days estimated by eye. ++++, heavy growth; −, no growth; +, light growth.

E. coli Strains—The wild-type strain was pBWU13.4/DK8 (37). Mutant strain βR246A/DK8 was as described previously (11). New mutant strains were constructed as below.

Construction of Mutant Strains of E. coli—Mutagenesis was by the method of Vandeyar *et al.* (38). For βN243R/βR246A and βN243R mutants, the template for oligonucleotide-directed mutagenesis was M13mp18 containing the HindIII-XbaI fragment from pSN6. pSN6 is a plasmid containing the βY331W mutation from plasmid pSWM4 (18) introduced on a SacI-EagI fragment into pBWU13.4 (37), which expresses all the ATP synthase genes. Mutagenic oligonucleotides were as follows: βN243R/βR246A, GCTGTTCGTTGACCGCATCTATGCATACACCCTGGCCG (where the underlined bases introduce the mutation and a new Nsi1 restriction site); βN243R, GTGTTCGTCGACCGCATCTATCGTTAC (where the underlined bases introduce the mutation and a new SalI 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 pZA8 (βN243R/βR246A/βY331W) and pZA15 (βN243R/βY331W). Each plasmid was transformed into strain DK8 (39) containing a deletion of ATP synthase genes for expression of the mutant enzymes. For αF291R, αF291R/βR246A, and αF291R/βN243R mutants, the template for oligonucleotide-directed mutagenesis was M13mp18 containing the SphI-SalI fragment from pSN6. The mutagenic oligonucleotide for αF291R was: CGGGCGACGTCCGCTACCTCCACTCTCG (where the underlined bases introduce the mutation and a new Aat2 restriction site). DNA sequencing was performed to confirm the presence of mutations and absence of undesired changes in sequence. The mutation was transferred to pSN6 on a XhoI-PmlI fragemt generating the new plasmid pZA10 (αF291R/βY331W). For new plasmid pZA9 (αF291R/βR246A/βY331W) a XhoI-PmlI fragment was transferred to pZA7 (11). For new plasmid pZA16 (αF291R/βN243R/βY331W) a SacI-EagI fragment was transferred from plasmid pZA15 to plasmid pZA10. Each plasmid was transformed into strain DK8 (39) containing a deletion of ATP synthase genes for expression of the mutant enzymes. It may be noted that all of the new mutant strains contained the βY331W mutation, which is valuable for measurement of nucleotide binding parameters (18) and does not affect function significantly. While it was not utilized in this work, the Trp mutation was included for possible future use.

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. Membranes (0.2–2.0 mg/ml) were reacted with NBD-Cl for 60 min in the dark, at room temperature, in 50 mM Tris/SO_4, pH 8.0, 2.5 mM $MgSO_4$, then 50-μl aliquots were transferred to 1

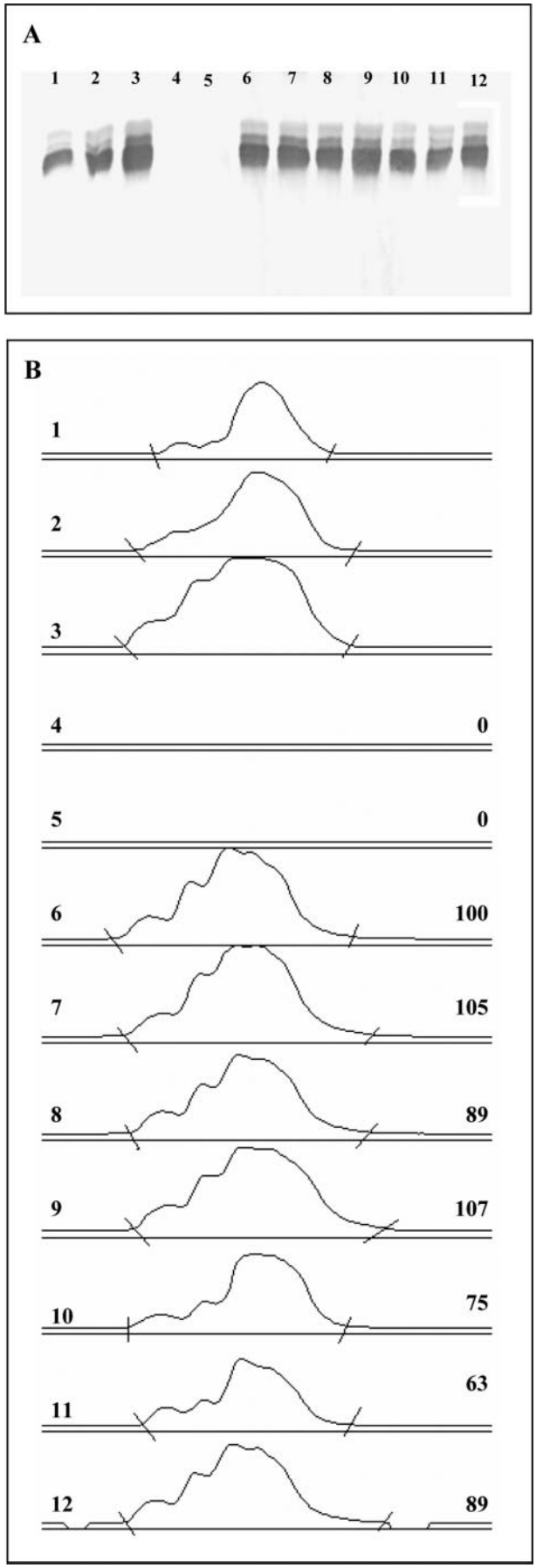

FIG. 1. **Immunoblotting and densitometry of mutant and wild-type membrane preparations with anti-F_1-α antibody.** Membrane preparations (4 μg of protein), prepared and washed as described under "Materials and Methods," were run on 10% SDS-polyacrylamide gels together with purified wild-type F_1 (0.1–0.4 μg) as reference. Protein bands were transferred to nitrocellulose and immunoblotted using anti-F_1-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_1, 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 βR246A (*lane 7*), βN243R/βR246A (*lane 8*), αF291R/βR246A (*lane 9*), αF291R (*lane 10*), βN243R (*lane 11*), and βN243R/αF291R (*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.

TABLE II
ATPase activity and proton pumping in mutant membranes

Mutation	ATPase activity[a]	Proton pumping activity[b]
Wild-type[c]	14.7 ± 2.21 (24)	84
Null mutant (1)[c]	0.0013 ± 0.0008 (16)[d]	0
Null mutant (2)[c]	0.0016 ± 0.0004 (16)[d]	0
βR246A	0.050 ± 0.0059 (16)	0
βN243R	0.023 ± 0.0037 (20)	0
βN243R/R246A	0.016 ± 0.0046 (24)	0
αF291R	0.035 ± 0.0051 (23)	0
αF291R/βR246A	0.52 ± 0.11 (24)	3
αF291R/βN243R	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 (βR246A, βN243R/βR246A) 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 P_i (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 Fluoroscandium—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-μl 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 βArg-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 βArg-246 to Ala abrogates P_i binding (11). We introduced Arg at two residues located close to βArg-246, namely βAsn-243 and αPhe-291, to generate the new mutants βN243R, βN243R/βR246A, αF291R, and αF291R/βR246A. These mutants are designed to test the

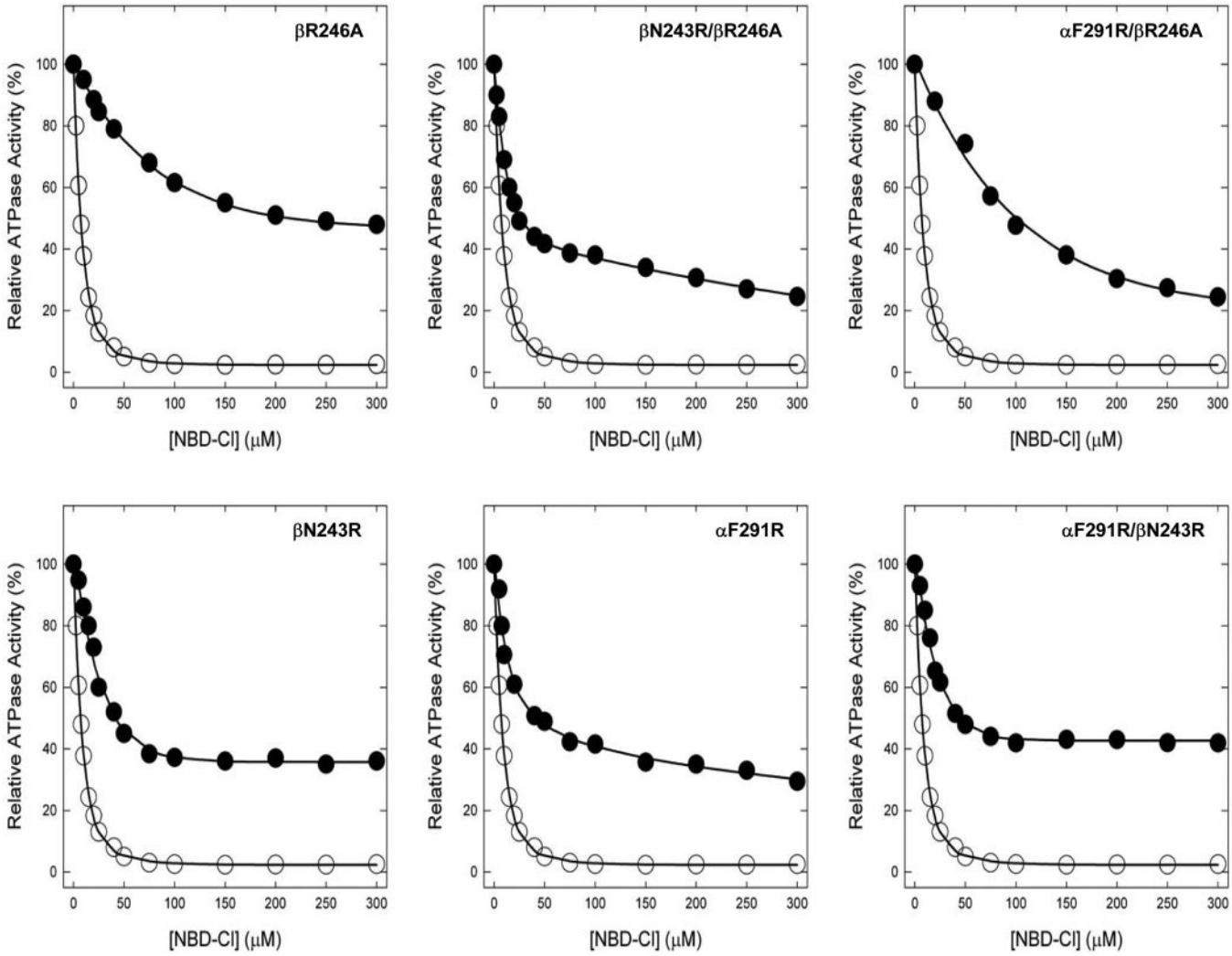

FIG. 2. **Inhibition of ATPase activity in membrane-bound wild-type and mutant ATP synthase by NBD-Cl.** Membranes were preincubated for 60 min at 23 °C with the indicated concentration of NBD-Cl, then aliquots were added to 1 ml of assay buffer and ATPase activity determined. For details see "Materials and Methods." ○, wild type; ●, mutant, as indicated in the separate panels. Each data point represents average of at least four experiments, using two independent membrane preparations of each mutant. In each case the "100%" point is the uninhibited rate of ATPase as shown in Table II.

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 βR246A 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 P_i binding by mutant and wild-type ATP synthase can be assayed using either membrane preparations or purified F_1. 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 F_1 as reference) established that all the mutant membrane preparations had bands running at the position of F_1-α and F_1-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 F_1 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-F_1-α subunit is shown in Fig. 1*A*. Purified F_1 (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. 1*B*, 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

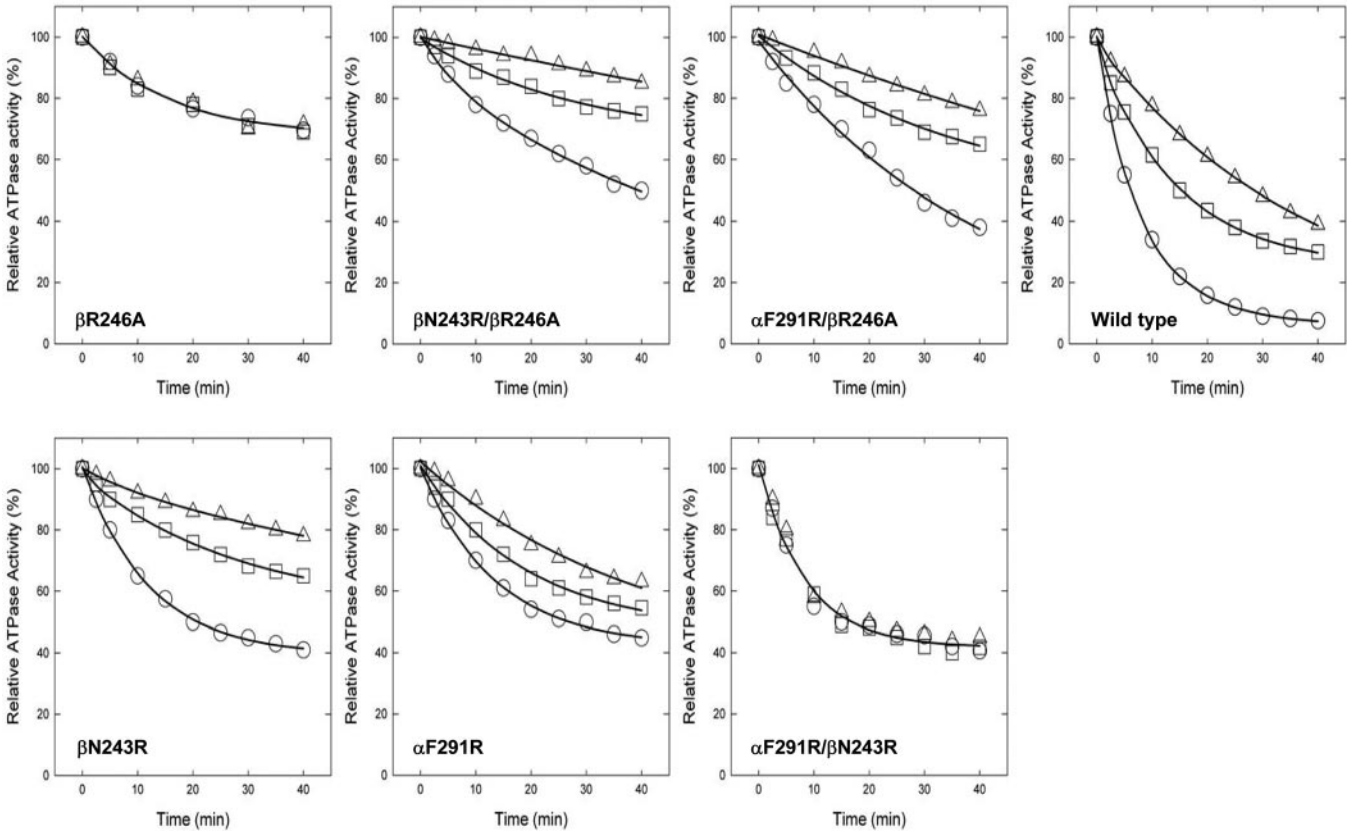

FIG. 3. **Protection by P_i of ATPase activity in wild-type and mutant membranes from inactivation by NBD-Cl.** Membranes were preincubated with P_i at zero, 2.5, or 10 mM concentration as shown, for 60 min at 23 °C. Then NBD-Cl (125 μM) was added and aliquots withdrawn for assay at time intervals as shown. ATPase activity remaining is plotted against time of incubation with NBD-Cl. ○, no P_i added; □, 2.5 mM P_i; △, 10 mM P_i. Each data point represents the average of four different experiments using two independent membrane preparations of each mutant.

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. Immunoblotting using anti-F_1-β 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 P_i binding site (αF291R, βN243R, αF291R/βN243R) in otherwise wild-type background (*i.e.* with βArg-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 βArg-243 in presence of βAla-246 (βN243R/βR246A) did not restore ATPase activity. Third, insertion of αArg-291 in presence of βAla-246 (αF291R/βR246A) did significantly restore ATPase activity (by 10-fold over βR246A 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 αF291R/βR246A the partial "rescue" of βR246A 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 βR246A 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 μM NBD-Cl, then added a further pulse of NBD-Cl, equivalent to additional 200 μM 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 for 1 h at room temperature. This also occurred in wild type and is known to be due to release of the NBD-adduct from βTyr-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

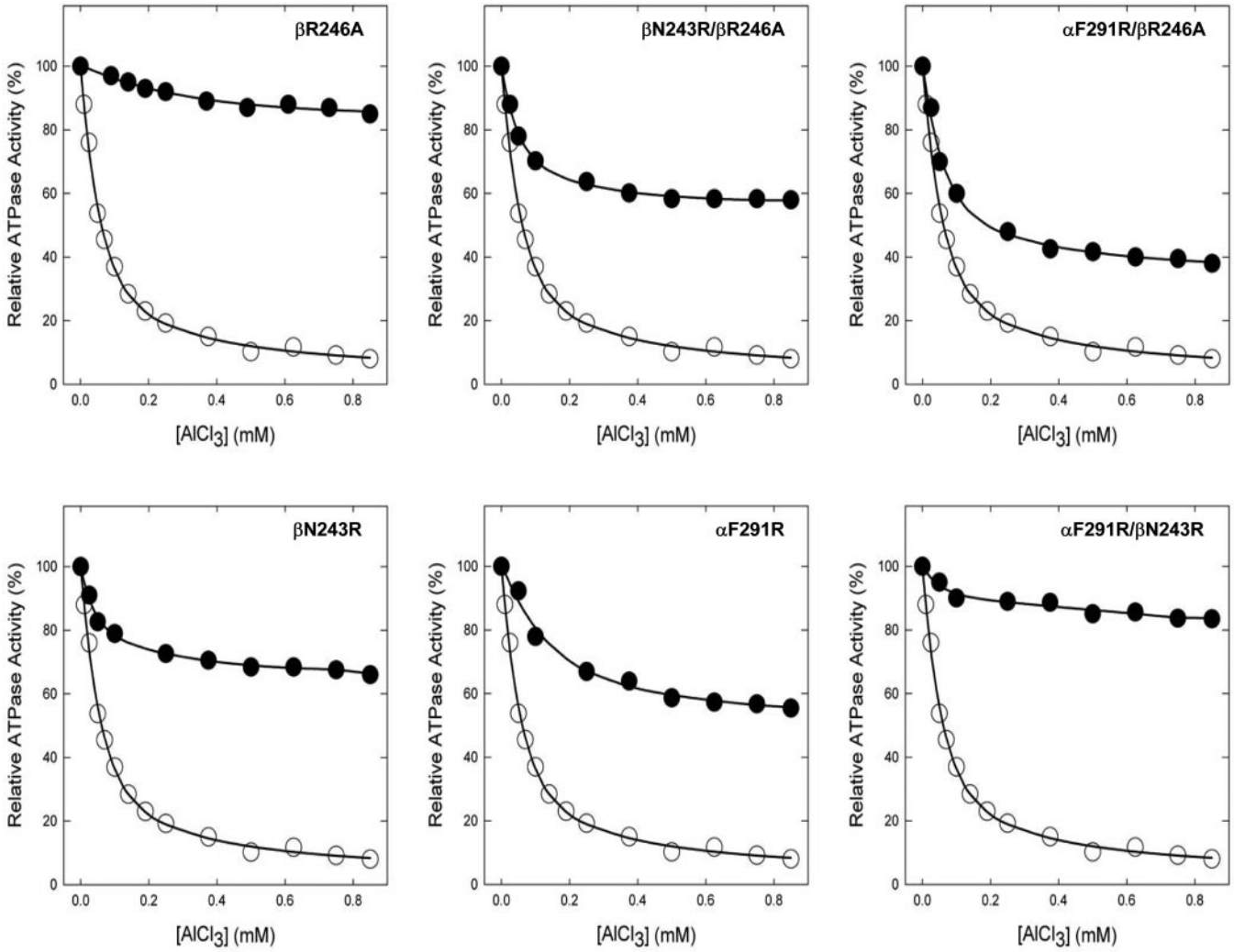

FIG. 4. **Inhibition of membrane ATPase activity from mutant and wild-type ATP synthase enzymes by fluoroaluminate.** Membranes were preincubated for 60 min at 23 °C with 1 mM MgADP, 10 mM NaF, and the indicated concentration of $AlCl_3$, then aliquots were added to 1 ml of assay buffer and ATPase activity determined. For details see "Materials and Methods." ○, wild type; ●, mutant, as indicated in the separate panels. Each data point represents average of duplicate experiments.

MgADP binding in catalytic site βE where NBD-Cl reacts (11, 23).

Inhibition of ATPase Activity by NBD-Cl in Membranes Containing Mutant ATP Synthase; Protection by P_i—Fig. 3 demonstrates that P_i protected well against NBD-Cl inhibition of ATPase activity in wild type but not in βR246A mutant, confirming previous work (11). It is seen that mutants βN243R/βR246A and αF291R/βR246A both showed clear protection by P_i. It is apparent that insertion of an Arg at position β-243 or α-291 compensates for the loss of the natural Arg at β-246 in P_i binding. P_i binding was retained in αF291R and βN243R. Therefore introduction of one extra Arg did not interrupt P_i binding. However, introduction of two extra Arg (αF291R/βN243R mutant) prevented P_i 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 βR246A and wild-type membranes, and it may be noted that the data are very similar to the parallel data reported previously (11) where purified F_1 was used. Wild type was very strongly inhibited (>95%), and βR246A was inhibited by 15% at the highest $AlCl_3$ concentration. Inclusion of "replacement" Arg in βN243R/βR246A or αF291R/βR246A mutants increased inhibition markedly, to maximally 42 and 62%, respectively. Inclusion of one additional Arg (βN243R, αF291R) gave maximal inhibition of 34 and 45%, respectively, *i.e.* less than wild type by far, and less than when in combination with βAla-246 but higher than βAla-246 alone. Inclusion of two additional Arg (αF291R/βN243R) gave 17% inhibition, similar to βR246A. 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 βR246A, 28% for βN243R/βR246A, 62% for αF291R/βR246A, 24% for βN243R alone, 49% for αF291R alone, and zero for αF291R/βN243R.

DISCUSSION

P_i binding is a primary step in ATP synthesis by ATP synthase, so that understanding the molecular basis of P_i 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 P_i binding in the βE catalytic site of *E. coli* ATP synthase (11, 26). X-ray crystallography structures of ATP synthase catalytic sites containing ADP with bound AlF_3 (27) or SO_4^{2-} (28) as phosphate analogs are consistent with these conclusions. To-

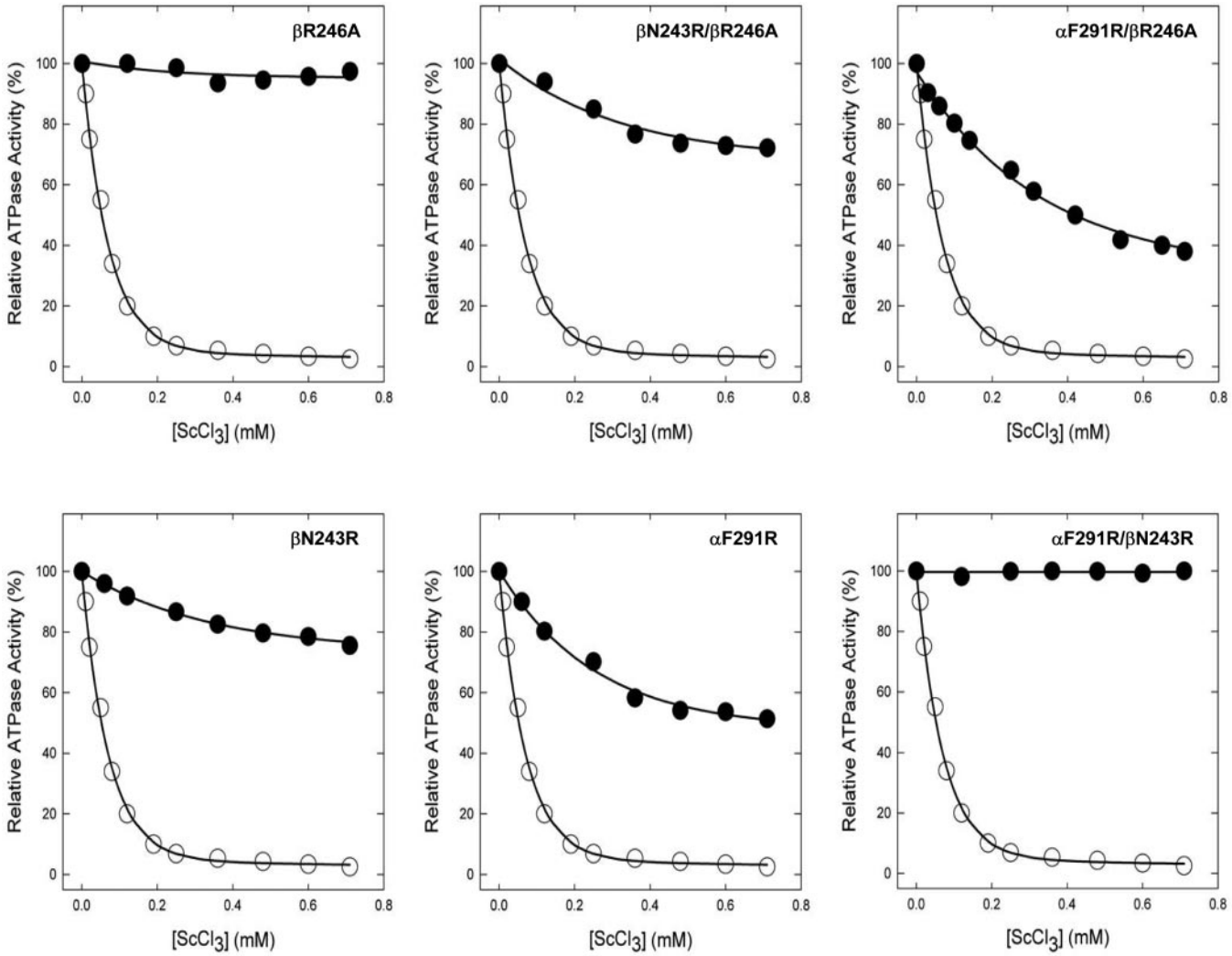

FIG. 5. **Inhibition of membrane ATPase activity from mutant and wild-type ATP synthase enzymes by fluoroscandium.** Membranes were preincubated for 60 min at 23 °C with 1 mM MgADP, 10 mM NaF, and the indicated concentration of $ScCl_3$, then aliquots were added to 1 ml of assay buffer and ATPase activity determined. For details see "Materials and Methods." ○, wild type; ●, mutant, as indicated in the separate panels. Each data point represents average of duplicate experiments.

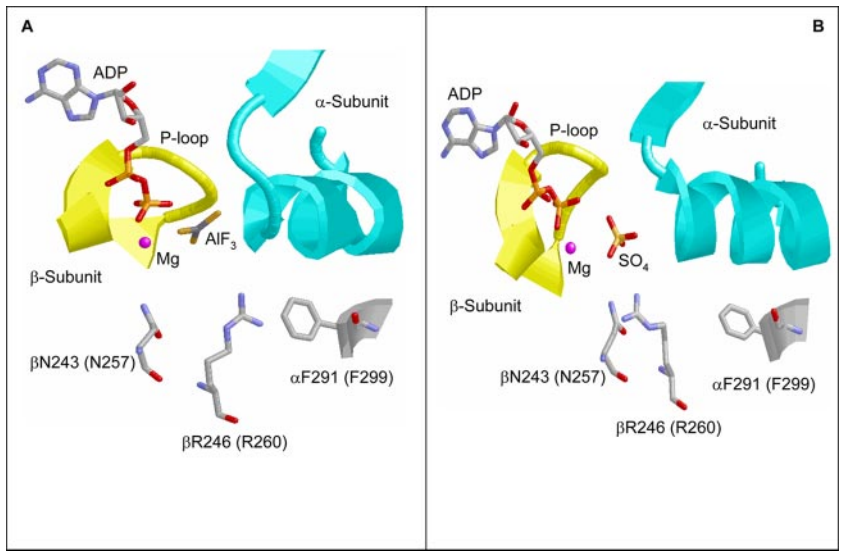

FIG. 6. **Spatial relationship of residues βAsn-243, βArg-246, and αPhe-291 to AlF_3 and SO_4^{2-} bound in catalytic sites of ATP synthase.** Rasmol software was used to generate these figures from the x-ray structures. *A*, AlF_3 in the βDP catalytic site of AlF_3-inhibited enzyme (27). *B*, SO_4^{2-} in the βE site of AlF_4^--inhibited enzyme (28). *E. coli* residue numbering is shown, with corresponding bovine mitochondrial residue numbers in *parentheses*.

gether 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 P_i binding pocket, close to βArg-246, prevented P_i binding. This suggested that modulation of charge in the P_i binding site could be used to illuminate the molecular mechanism of P_i binding. It is established that Arg residues occur particularly commonly in P_i binding sites in proteins (30), therefore varying the number

TABLE III
Distances within the P_i binding subdomain of ATP synthase catalytic sites

Distances shown are between the nearest atom of the residue and the P_i analog, in Ångstroms (Å), as determined by x-ray crystallography (27, 28). Values in parentheses are speculative distances calculated for mutant residues (also in parentheses) calculated using the Deep View Swiss-Pdb Viewer, Version 3.7 (N. Guex, A. Diemand, M.C. Peitsch, and T. Schwede (2004)) at www.us.expasy.org/spdbv/mainpage.html).

Residue	AlF_3	SO_4^{2-}
αPhe-291 (αArg-291)	7.6 (6.0)	9.6 (8.6)
βAsn-243 (βArg-243)	5.4 (2.6)	3.9 (1.3)
βArg-246 (βAla-246)	5.0 (9.4)	3.7 (8.6)

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^{2-}-containing catalytic sites (nearest atom distances quoted) and close to either AlF_3 or SO_4^{2-} (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^{2-}-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^{2-} as determined by x-ray crystallography (27, 28), together with speculative distances (in parentheses) calculated for mutant residues βAla-246, βArg-243 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-243 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-fluoroaluminate 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.

REFERENCES

1. Senior, A. E., and Weber, J. (2004) *Nat. Struct. Mol. Biol.* **11,** 110–112
2. Weber, J., and Senior, A. E. (2003) *FEBS Lett.* **545,** 61–70
3. Senior, A. E., Nadanaciva, S., and Weber, J. (2002) *Biochim. Biophys. Acta* **1553,** 188–211
4. Noji, H., and Yoshida, M. (2001) *J. Biol. Chem.* **276,** 1665–1668
5. Leslie, A. G. W., and Walker, J. E. (2000) *Philos. Trans. R. Soc. Lond. B Biol. Sci.* **355,** 465–472
6. Rosing, J., Kayalar, C., and Boyer, P. D. (1977) *J. Biol. Chem.* **252,** 2478–2485
7. Boyer, P. D. (1989) *FASEB J.* **3,** 2164–2178
8. Al-Shawi, M. K., and Senior, A. E. (1992) *Biochemistry* **31,** 886–891
9. Al-Shawi, M. K., Ketchum, C. J., and Nakamoto, R. K. (1997) *Biochemistry* **36,** 12961–12969
10. Masaike, T., Muneyuki, E., Noji, H., Kinosita, K., and Yoshida, M. (2002) *J. Biol. Chem.* **277,** 21643–21649
11. Ahmad, Z., and Senior, A. E. (2004) *J. Biol. Chem.* **279,** 31505–31513
12. Yasuda, R., Masaike, T., Adachi, K., Noji, H., Itoh, I., and Kinosita, K. (2003) *Proc. Natl. Acad. Sci. U. S. A.* **100,** 9314–9318
13. Shimabukuro, K., Yasuda, R., Muneyuki, E., Hara, K., Kinosita, K., and Yoshida, M. (2003) *Proc. Natl. Acad. Sci. U. S. A.* **100,** 14731–14736
14. Nishizaka, T., Oiwa, K., Noji, H., Kimura, S., Muneyuki, E., Yoshida, M., and Kinosita, K. (2004) *Nat. Struct. Mol. Biol.* **11,** 142–148
15. Hirono-Hara, Y., Ishizuka, K., Kinosita, K., Yoshida, M., and Noji, H. (2005) *Proc. Natl. Acad. Sci. U. S. A.* **102,** 4288–4293
16. Penefsky, H. S. (1977) *J. Biol. Chem.* **252,** 2891–2899
17. Kasahara, M., and Penefsky, H. S. (1978) *J. Biol. Chem.* **253,** 4180–4187
18. Weber, J., Wilke-Mounts, S., Lee, R. S. F., Grell, E., and Senior, A. E. (1993) *J. Biol. Chem.* **268,** 20126–20133
19. Löbau, S., Weber, J., and Senior, A. E. (1998) *J. Biol. Chem.* **37,** 10846–10853
20. Weber, J., and Senior, A. E. (1995) *J. Biol. Chem.* **270,** 12653–12658
21. Ferguson, S. J., Lloyd, W. J., Lyons, M. H., and Radda, G. K. (1975) *Eur. J. Biochem.* **54,** 117–126
22. Ferguson, S. J., Lloyd, W. J., and Radda, G. K. (1975) *Eur. J. Biochem.* **54,** 127–133
23. Orriss, G. L., Leslie, A. G. W., Braig, K., and Walker, J. E. (1998) *Structure (Lond.)* **6,** 831–837
24. Perez, J. A., Greenfield, A. J., Sutton, R., and Ferguson, S. J. (1986) *FEBS Lett.*

198, 113–118
25. Ahmad, Z., and Senior, A. E. (2004) *J. Biol. Chem.* **279,** 46057–46064
26. Ahmad, Z., and Senior, A. E. (2005) *FEBS Lett.* **579,** 523–528
27. Braig, K., Menz, R. I., Montgomery, M. G., Leslie, A. G. W., and Walker, J. E. (2000) *Structure* (*Lond.*) **8,** 567–573
28. Menz, R. I., Walker, J. E., and Leslie, A. G. W. (2001) *Cell* **106,** 331–341
29. Penefsky, H. S. (2005) *FEBS Lett.* **579,** 2250–2252
30. Copley, R. R., and Barton, G. J. (1994) *J. Mol. Biol.* **242,** 321–329
31. Senior, A. E., Langman, L., Cox, G. B., and Gibson, F. (1983) *Biochem. J.* **210,** 395–403
32. Senior, A. E., Latchney, L. R., Ferguson, A. M., and Wise, J. G. (1984) *Arch. Biochem. Biophys.* **228,** 49–53
33. Taussky, H. H., and Shorr, E. (1953) *J. Biol. Chem.* **202,** 675–685
34. Perlin, D. S., Cox, D. N., and Senior, A. E. (1983) *J. Biol. Chem.* **258,** 9793–9800
35. Laemmli, U. K. (1970) *Nature* **227,** 680–685
36. Rao, R., Perlin, D. S., and Senior, A. E. (1987) *Arch. Biochem. Biophys.* **255,** 309–315
37. Ketchum, C. J., Al-Shawi, M. K., and Nakamoto, R. K. (1998) *Biochem. J.* **330,** 707–712
38. Vandeyar, M., Weiner, M., Hutton, C., and Batt, C. (1988) *Gene* (*Amst.*) **65,** 129–133
39. Klionsky, D. J., Brusilow, W. S. A., and Simoni, R. D. (1984) *J. Bacteriol.* **160,** 1055–1060