Journal of Bioenergetics and Biomembranes, Vol. 37, No. 6, December 2005 (© 2005)
DOI: 10.1007/s10863-005-9486-8

Identification of Phosphate Binding Residues of *Escherichia coli* ATP Synthase

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Four positively-charged residues, namely βLys-155, βArg-182, βArg-246, and αArg-376 have been identified as Pi binding residues in *Escherichia coli* ATP synthase. They form a triangular Pi binding site in catalytic site βE where substrate Pi initially binds for ATP synthesis in oxidative phosphorylation. Positive electrostatic charge in the vicinity of βArg-246 is shown to be one important component of Pi binding.

KEY WORDS: Oxidative phosphorylation; ATP synthesis; ATP synthase; catalytic site βE; Pi binding subdomain; Pi binding residues.

INTRODUCTION

The residues responsible for specific binding of phosphate (Pi) into the catalytic sites of ATP synthase in oxidative phosphorylation are of considerable interest. First and foremost, Pi is a required substrate for ATP synthesis. Secondly, Pi binding is thought to be linked to subunit rotation energized by the proton gradient, and thirdly, expedited capture of Pi in an empty catalytic site provides in principle a mechanism by which the enzyme can bind ADP in the face of a seemingly prohibitive ATP/ADP concentration ratio in the surrounding medium (Senior *et al*., 2002; Weber and Senior, 2003). In papers published in 2004 and 2005 we have identified the phosphate binding residues and characterized the role of positive charge in Pi binding. That work is summarized here.

RESULTS AND DISCUSSION

Escherichia coli remains the only system in which mutagenesis of ATP synthase can be combined conveniently with assays of growth and oxidative phosphorylation *in vivo*, and with assays of ATPase, ATP-driven proton pumping, and ATP synthesis rates *in vitro*. As this project began there was no available assay of Pi binding to *E. coli* enzyme. Earlier attempts to measure Pi binding to purified F_1 by use of [^{32}P]Pi (Al-Shawi and Senior, 1992) or by competition with ATP or AMPPNP in fluorescence assays of nucleotide binding (Löbau *et al*., 1998; Weber and Senior, 1995) failed to detect appreciable Pi binding at physiological Pi concentration. Thus, we turned to the assay devised by Perez *et al*. (1986) in which the protection afforded by Pi against inhibition of ATPase activity induced by covalent reaction with 7-chloro-4-nitrobenzo-2-oxa-1,3,-diazole (NBD-Cl) provides the measure of Pi binding.

The three catalytic sites located on the F_1 sector of ATP synthase have been designated βDP, βTP, and βE by X-ray crystallographers (Leslie and Walker, 2000). βE is the empty site into which Pi must initially bind for ATP synthesis. Fortuitously, NBD-Cl was shown to react only in site βE (Orriss *et al*., 1998). Thus the assay of Perez *et al*. (1986) avoids ambiguities by measuring Pi binding specifically in site βE. Their original work used mitochondrial inner membrane preparations; here we applied the assay successfully to both purified F_1 and plasma membrane vesicle preparations from *E. coli*, with interchangeable results. Any nucleotide resident in catalytic sites was first removed by repeated centrifuge column elution (F_1)

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Abbreviations: DTT, dithiothreitol; NBD-Cl,7-chloro-4-nitro-benzo-2-oxa-1,3,-diazole.

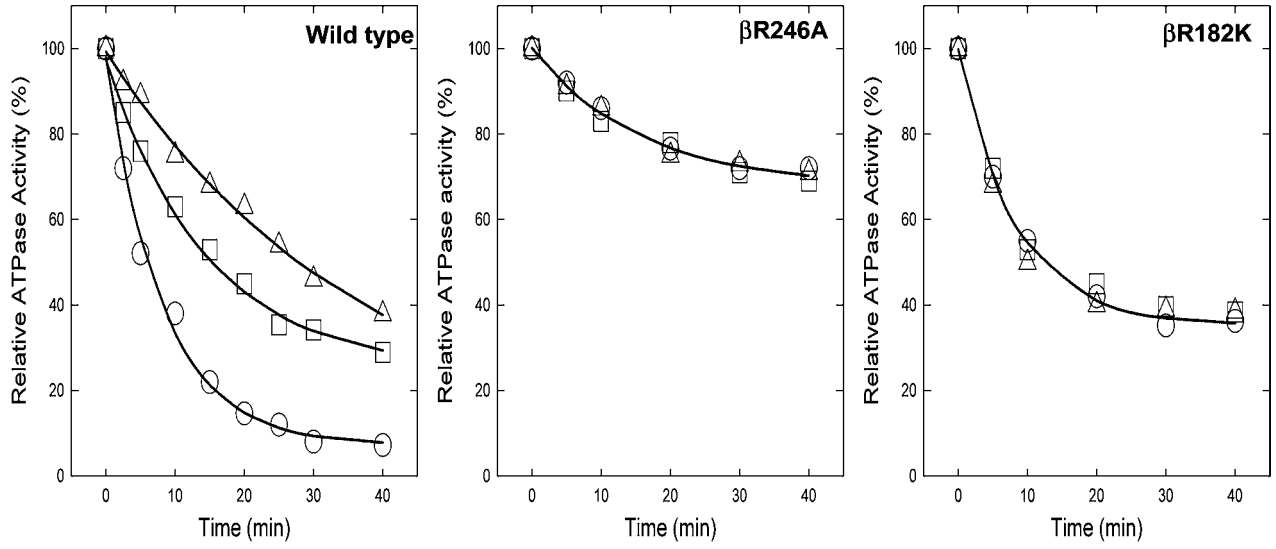

Fig. 1. Inhibition of ATPase activity of wild-type and mutant purified *E. coli* F_1 by NBD-Cl and protection by Pi. Enzymes were preincubated with Pi for 1 h at room temperature, then 100 μM NBD-Cl was added. Aliquots were withdrawn for ATPase assay at time intervals shown. ATPase activity remaining is plotted against time of incubation with NBD-Cl. (○), no Pi added; (□), 2.5 mM Pi; (△), 10 mM Pi. As noted in the text, the same results were seen with membrane preparations.

or centrifugal washing (membranes). We found that in wild type, NBD-Cl inhibited ATPase potently (≥95%) and inhibition was completely reversed by subsequent incubation with dithiothreitol (DTT). From previous work it is known that DTT restores activity by releasing NBD from its covalent reaction site (Ferguson *et al*., 1975; Orriss *et al*., 1998,); in *E. coli* this is residue βTyr-297. We also found that MgADP protected against NBD-Cl inhibition in wild-type with EC_{50} ∼4 mM, consistent with reaction occurring in catalytic site βE.

Our strategy for identifying Pi binding residues was to mutagenize candidate residues appropriately and test the resulting effects on Pi binding using the NBD-Cl inhibition assay. Earlier biochemical evidence describing effects of mutations on rate constants for Pi binding/release in unisite catalysis (Al-Shawi *et al*., 1989; Senior and Al-Shawi, 1992) and on transition state formation (Senior *et al*., 2000) provided one source of candidate residues. X-ray structures of mitochondrial F_1 containing the phosphate analogs AlF_3 (Braig *et al*., 2000) and SO_4^{2-} (Menz *et al*., 2001) bound in catalytic sites were another, important, source. Residues βLys-155, βArg-182, βAsn-243, βArg-246, and αArg-376 (*E. coli* numbering) were targeted. Details of the experiments may be found in Ahmad and Senior (2004a, 2005a); in the following we present a brief summary.

ATPase activity of all mutant enzymes was much lower than that of wild type. With mutants, NBD-Cl reaction was found to inhibit, or in some instances, to enhance ATPase activity, but the effect was in all cases DTT-reversible, and MgADP-protectable with EC_{50} ∼4 mM. Often NBD-Cl inhibition was only partial, with the fully-reacted mutant enzyme retaining residual ATPase activity. Figure 1 shows an experiment in which Pi protection against NBD-Cl inhibition was measured in wild type and two mutants. NBD-Cl was added at time zero, in the presence of 0, 2.5, or 10 mM Pi, and ATPase was assayed over time. The results for wild type, showing significant protection by Pi, are similar to those seen originally by Perez *et al*. (1986). Mutants βR246A and βR182K, on the other hand, showed no protection, indicating that the mutations had abrogated Pi binding. Similar results were found with βR246K, βR246Q, βK155Q, βR182Q, and αR376Q. Thus, residues βLys-155, βArg-182, βArg-246, and αArg-376 are identified as Pi binding residues in catalytic site βE. BLAST searches showed that all four are totally conserved. The four residues form a triangular Pi binding site. Figure 2(A) shows their arrangement around bound SO_4^{2-} in the "half-closed" βE site of AlF_4^--inhibited F_1 (Menz *et al*., 2001) and Fig. 2(B) shows their very similar arrangement in the completely empty βE site in the AlF_3-inhibited enzyme (Braig *et al*., 2000).

From the X-ray structures containing AlF_3 or SO_4^{2-}, it would appear that residue βAsn-243 could well be involved in Pi binding since it is located close to the bound Pi analogs. However, with mutant βN243A, Pi protected well against NBD-Cl inhibition (Ahmad and

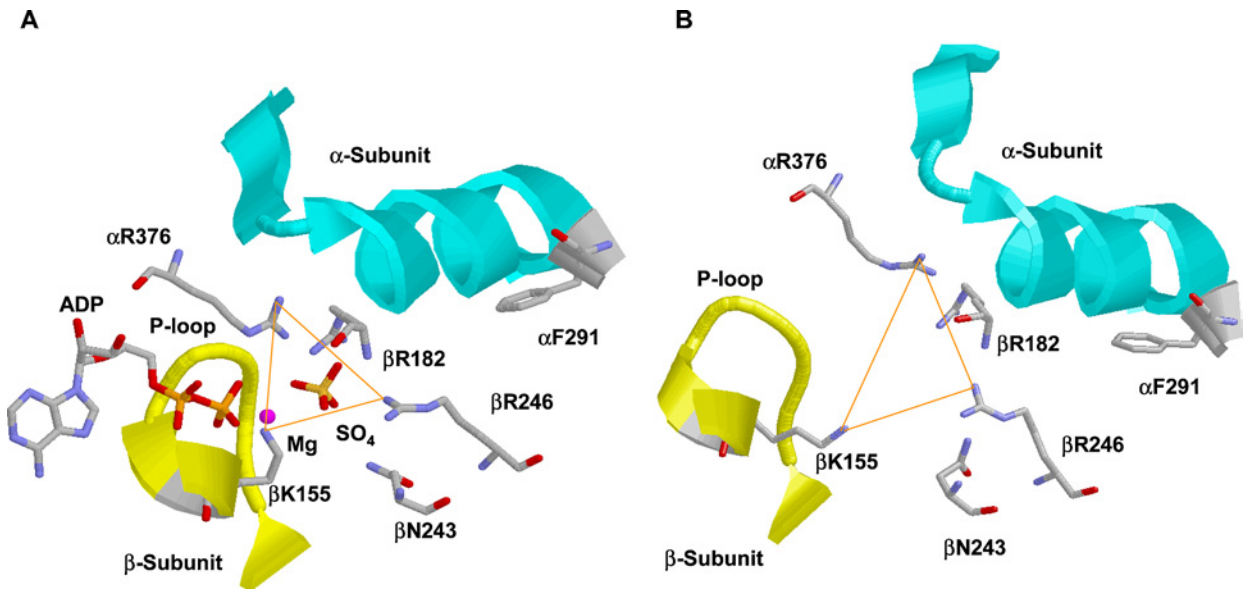

Fig. 2. The Pi binding site in ATP synthase. Residues βLys-155, βArg-182, βArg-246, and αArg-376 form a triangular binding site for Pi. (A.) The "half-closed" βE site of AlF_4^--inhibited bovine mitochondrial F_1 (Menz *et al*., 2001). This site contains bound SO_4^{2-} and MgADP. (B.) The completely empty βE site in bovine mitochondrial AlF_3-inhibited F_1 (Braig *et al*., 2000).

Senior, 2004b), from which we conclude that residue βAsn-243 is not required for Pi binding in catalytic site βE. On the other hand, mutant βN243D did abrogate Pi binding. A likely reason for this finding is that negative charge introduced by the Asp substitution at β-243 neutralizes positive charge of the very close βArg-246 (see Fig. 2).

To test whether positive charge *per se* in the vicinity of βArg-246 was important, we introduced new Arg residues into mutant βR246A (Ahmad and Senior, 2005b). As noted above, removal of natural βArg-246 in mutant βR246A abrogates Pi binding; restoration of Pi binding was achieved by mutagenesis of either residue βAsn-243 or αPhe-291 to Arg. Both residues are located in the Pi binding subdomain close to βArg-246 in X-ray structures (Fig. 2). This result showed that positive charge is an important component of initial Pi binding in site βE. Insertion of one extra Arg at β-243 or α-291 in the presence of βArg-246 retained Pi binding, but insertion of two extra Arg at both positions simultaneously in the presence of βArg-246 abrogated Pi binding. While, as noted above, insertion of Arg at β-243 or α-291 did rescue Pi binding in βR246A, nevertheless ATPase activity, ATP-driven proton pumping, and ATP synthesis *in vivo* were not by any means restored to normal levels, showing that specific stereochemical interactions of catalytic site Arg residues are of paramount importance. Notably, transition state stabilization, lost in βR246A, was not fully-restored in either βN243R/βR246A or αF291R/βR246A.

Summarizing, we have identified residues βLys-155, βArg-182, βArg-246, and αArg-373 as residues required for initial Pi binding in catalytic site βE during ATP synthesis by ATP synthase. Together they form a triangular Pi binding site. Residue βAsn-243, although close to this site, is not required for Pi binding. Positive electrostatic charge in the vicinity of βArg-246 is an important component of initial Pi binding.

ACKNOWLEDGMENTS

Supported by NIH grant GM25349 to AES. We thank Erich Huang and Sarah Lockwood for outstanding technical assistance.

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