Intragenic Suppressors of P-loop Mutations in the β-Subunit of the Mitochondrial ATPase in the Yeast Saccharomyces cerevisiae*

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Three intragenic second-site suppressors, P353L, T237I, and L390F, were identified that suppressed two mutations in, and one adjacent to, the P-loop in the β-subunit of the yeast F1-ATPase. The crystal structure of bovine F1-ATPase (Abrahams, J. P., Leslie, A. G. W., Lutter, R., and Walker, J. E. (1994) Nature 370, 621–628) shows that these suppressor residues are located in the nucleotide-binding domain. Specific hypotheses have been formulated that suggest the conformational coupling of the P-loop with the suppressor sites. P353L is in a "catch" region, which forms unique interactions with the γ-subunit in the three different conformational states of the catalytic site. The identification of this suppressor mutation demonstrates genetically that the catch region is conformationally coupled to the P-loop. T237I is shown to interact with Lys-209, which occurs just after the P-loop. This suggests that this interaction changes the conformation of the P-loop to suppress the initial mutation. L390F interacts with Ala-181, which is adjacent to the P-loop. The mechanism of this suppression is suggested to occur through the interactions of L390F with Ala-181. These results identify critical interactions that modulate the structure of the P-loop and thus the biochemistry of the enzyme.

The mitochondrial ATP synthase is the major enzyme responsible for the aerobic synthesis of ATP. The ATP synthase is composed of a water-soluble portion, the F1-ATPase (EC 3.6.1.34), and a membrane portion, F0. The F1-ATPase has a subunit composition of α3β3γ6ε with an overall mass of ~360,000 Da (1, 2). The recent determination of the high resolution (2.8 Å) structure of the bovine F1-ATPase (3) and prior biochemical and mutagenesis studies (for review, see Ref. 4) indicate that the β-subunits, together with small contributions from the α-subunits, compose the active sites of the enzyme.

The binding change hypothesis for ATP synthesis proposed by Boyer et al. (5–7) suggests that the energy-requiring step for ATP synthesis is the release of newly synthesized ATP and not the phosphorylation step. The hypothesis proposes that three catalytic sites in F1 participate in the synthesis of ATP by a mechanism that involves conformational changes mediating changes in affinity of the active sites for ATP. The binding change hypothesis is supported by a number of biochemical studies (for review, see Ref. 6) and recently by the high resolution crystal structure of the protein (3). The crystal structure indicates that the three active sites are not identical: one site is occupied by ADP (βTP), one site by AMP-PNP (βTP), and one site is vacant (βE). The differences in βTP, βTP, and βE are also demonstrated by large conformational differences in the active-site domain of each β-subunit, including the structure and location of the P-loop motifs.

The P-loop motif (8, 9) is present in many nucleotide-binding proteins (9, 10). The primary sequence of the P-loop in the β-subunit of F1 is Gly-Gly-Ala-Gly-Val-Lys-Thr. Crystallographic studies indicate that the backbone structure of the P-loop is the same in many nucleotide-binding proteins: p21ras elongation factor Tu, myosin, RecA, adenylate kinase, and the ATPase (3, 11–15). In p21ras, the P-loop has extensive hydrogen bonding with the α-, β-, and γ-phosphates of Mg2+-GMP-PNP (12). Biochemical studies of mutants in Escherichia coli and yeast F1 indicate that the P-loop is critical for catalysis (16–19). Furthermore, recent studies in this laboratory indicated that the primary structural constraints of the P-loop in the β-subunit of the yeast ATPase correspond very well with the known structure of p21ras (19). As revealed by the crystal structure of F1, the P-loop is in a dramatically different conformation in βE as compared with βTP and βTP, which suggests that the conformation of the P-loop may explain the vacancy of βE for nucleotides. As such, changes in the position, geometry, and structure of the P-loop of the mitochondrial ATPase may, in part, be responsible for changes in the biochemistry of the active site during the catalytic reaction cycle.

This study was initiated to identify residues in the β-subunit that interact with the P-loop. Intragenic suppressors were isolated in strains with mutation codons coding for residues in, and immediately adjacent to, the P-loop of the β-subunit of F1. The primary structural constraints on these residues were postulated to be determined by steric interactions with other residues in the enzyme (19). This study has identified critical interacting residues in the β-subunit that appear to be important conformational links to the P-loop in the active site of the enzyme. These interactions may be important in the catalytic mechanism for the transition from the high to the low affinity conformation, or they may be important for modulating the activity of the enzyme to suit the needs of the particular organism.

MATERIALS AND METHODS

Strains and Growth Media

The yeast Saccharomyces cerevisiae strain DMY201 (19) (MATa, ade2-100, his3-1200, leu2-1Δ1, lys2-801, trp1-Δ36, ura3-52, atp2::leu2) containing plasmids with the mutant gene coding for the β-subunit of

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1The abbreviations used are: AMP-PNP, adenosine 5′-[(β,γ-iminotriphosphate); GMP-PNP, guanosine 5′-[(β,γ-iminotriphosphate).
the ATPase was used in this study. The bacterial strain XL1-Blue (Stratagene) was used throughout the study for the cloning procedures. Bacteria were infected with the helper phage VCSM13 (Stratagene) for the synthesis of single-stranded DNA.

The following media were used for the growth of the yeast strains: YPD (1% yeast extract, 2% peptone, 2% glucose), YPG (1% yeast extract, 2% peptone, 0.1% yeast nitrogen base without amino acids, 2% glucose). SD medium was supplemented with appropriate auxotrophic requirements at 20 mg/liter.

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The initial goal of this study was the isolation of intragenic suppressors of mutations at or adjacent to the P-loop, specifically at residues 192 and 194 and residue 198. These residues, as well as residues in the P-loop, are completely conserved between β-subunits of all F-type ATPases in the SWISS-PROT data base. Residues 192, 194, and 198 were postulated to

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Table I
Primary structural requirements at positions 192, 194, and 198 of the β-subunit

| Residue     | 192 | 194 | 198 |
|-------------|-----|-----|-----|
| Wild-type sequence | A   | V   | V   |
| Functional variants | P   | T   | C   |
|           | I   | T   |     |
| Dysfunctional variants | G   | G   | A   |
|           | S   | L   | K   |
|           | V   | M   | L   |
|           | N   | M   |     |
|           | Y   | S   |     |
| Nonfunctional variants | R   | R   | G   |

The table is derived from work presented earlier [19], except for Gly-194. The functional variants grow normally or almost normally on YPG medium as compared with the wild-type strain. The dysfunctional variants have growth phenotypes on YPG medium that are conditional and cold (18°C) or temperature (37°C)-sensitive or grow very slowly at 30°C. The nonfunctional variants do not grow on YPG medium at any of the three temperatures.

Fig. 1 shows the growth phenotypes of the original mutants, the mutants with a suppressor mutation, and the suppressor mutations in a wild-type background. Despite the fact that the suppressors were isolated at one temperature, all of the suppressor mutations complemented the defective growth phenotype at 18 and 30°C, while only T237I was effective at 37°C. These data indicate that the suppressor mutations do not generally provide an enzyme with completely wild-type properties.

The mechanism by which the suppressor mutation is transmitted to the P-loop in the active site of the enzyme is not clear. However, the suppressor mutations might act to alter the enzyme conformation or to stabilize the binding of the nucleotide. For V198S, the only revertants isolated, this low number of intragenic suppressors may be due to limitations imposed by the genetic code or to a severe limitation on the number of replacements that will suppress these mutations.

Table II
Suppressors obtained from strains with mutations at residues 192, 194, and 198

| Mutant | A192V | V194M | V194Y | V198S |
|--------|-------|-------|-------|-------|
| Phenotype | cs, ts, sg | cs, ts | cs, ts, sg |
| Mutagenesis | PCR, EMS | None | EMS | None |
| Temperature | 30°C | 37°C | 18°C | 30°C |
| Suppressor/ revertant | V192A | P353L | T237I | L390F |
|                   | GTC → GCC | CCT → CTT | ACT → ATT | TTA → TTC |

*PCR, polymerase chain reaction; EMS, ethyl methanesulfonate.*

The data in Table II indicate that Leu-390, Met-390, or Thr-390 may be important for the conformational changes observed in the catalytic site during the reaction cycle [19]. The steric interactions need not be limited to the most stable structure of the enzyme, but may occur during the reaction cycle, as in the transition state of the enzyme. Analysis of the crystal structure of bovine F1 indicates that residues 192 and 194 do not interact with any residues outside the P-loop, while residue 198 may interact with the adenine ring of the bound nucleotide [3].

Table I shows growth phenotypes of yeast with various replacements at positions 192, 194, and 198 in the β-subunit of the ATPase. The results described in Table I suggest that the size of the amino acid side chain is an important determining factor in the function of the enzyme. Particularly, position 198 appears to require a side chain that is about the size of Val. If the side chain is too small, such as Ala or Ser, or too large, such as Met or Lys, the enzyme is defective, and with Gly at this position, the enzyme is inactive. The crystal structure of bovine F1 indicates that the side chain of Val-198 is 3.8 Å from the adenine ring of the nucleotide. Thus, Val-198 may serve to position the nucleotide or to stabilize the binding of the nucleotide. In comparison, Tyr-378 is also 3.8 Å from the adenine ring and serves as a major determinant in stabilizing nucleotide binding [33]. As such, V198S would have a diminished ability to act in this manner, and suppression of V198S would need to compensate for this loss.

The requirements at positions 192 and 194 are not so apparent. However, a large number of replacements at these positions give dysfunctional phenotypes (temperature or cold sensitivity or slow growth), suggesting that the residues are not involved directly in the catalytic mechanism. Instead, these residues may serve structural roles or roles in the conformational coupling of the enzyme.

The dysfunctional mutants listed in Table I were used to isolate intragenic suppressors. Because suppressors may be allele-specific, attempts were made to isolate suppressors from each of the mutants listed as dysfunctional in Table I. Of these mutants, only A192V, V194Y, V194M, and V198S had strong enough phenotypes to isolate suppressors and provided a reversion or suppressor rate that allowed their isolation. Intragenic suppressors were isolated by a combination of methods as described under “Materials and Methods” and as summarized in Table II. The number and type of intragenic suppressors isolated were very limited. For mutations V194Y and V194M, only suppressors T237I and P353L were isolated, respectively. For V198S, L390F was the only suppressor mutation identified, whereas for A192V, only revertants were isolated. This low number of intragenic suppressors may be due to limitations imposed by the genetic code or to a severe limitation on the number of replacements that will suppress these mutations.

Table II shows the growth phenotypes of the original mutants, the mutants with a suppressor mutation, and the suppressor mutations in a wild-type background. Despite the fact that the suppressors were isolated at one temperature, all of the suppressor mutations complemented the defective growth phenotype at 18 and 30°C, while only T237I was effective at 37°C. These data indicate that the suppressor mutations do not generally provide an enzyme with completely wild-type properties.

If the amino acid residue corresponding to the suppressor forms a critical interaction with another residue, it would be expected that mutagenesis of this residue in an otherwise wild-type background would give a defective phenotype. This was tested by separating the suppressor mutation from the original P-loop mutation (Fig. 1). Suppressor L390F was defective at all temperatures, indicating that Leu-390 is an important residue in the F1-ATPase. In contrast, T237I and P353L showed only moderate effects on the growth phenotypes, indicating the replacements modify, but do not eliminate, the enzyme activity.

Another indicator of the critical importance of Leu-390 was demonstrated by site-directed mutagenesis of Leu-390 to Ala and Gly. Fig. 1 shows that strains with L390A or L390G have a negative growth phenotype on glycerol medium. These results indicate that Leu-390 makes important contacts with at least one other residue. Identification of the P-loop suppressor mutant L390F indicates that this interaction can be transmitted to the P-loop in the active site of the enzyme.

The allelic specificity of suppressor L390F was tested since this specificity should provide some information on the mechanism of the suppressor mutation. Specifically, the suppressor should show allelic specificity if it acts by reversing the effect of the primary mutation. This is in contrast to a mechanism whereby a suppressor improves the overall state of the enzyme, thereby overcoming the dysfunctional defect. Fig. 2 shows the growth phenotypes of the L390F mutation in the presence of 10 different residues at position 198. The results indicate that the suppressor mutation is allele-specific. At 30°C, a nearly wild-type growth phenotype for L390F was observed with Cys, Lys, Met, or Thr at position 198. However, at all three temperatures, L390F suppressed only Cys or Thr at position 198. Gly-198 was nonfunctional with either Leu or Phe at position 390. These data support the previous conclusion that Val-198 makes critical steric interactions with another residue or with the bound nucleotide.
formation was determined by analysis of the crystal structure of bovine F\textsubscript{1}-ATPase. Pro-353 is 20 Å from the \(\beta\)-phosphate of ADP in \(\beta\text{TP}\), so the mechanism of suppression must be by long-range conformational changes. Fig. 3A shows the proposed interaction pathway by which P353L could suppress V194M. Pro-353 is at the beginning of helix G of the nucleotide-binding domain of the ATPase and is in a “catch” region that forms unique interactions with the \(\gamma\)-subunit in the three different conformational states of the catalytic site. Helix G is also adjacent to \(\beta\)-sheet 8, which is adjacent to the P-loop. Arg-370 is located in \(\beta\)-sheet 8 and hydrogen-bonds with the carbonyl of Ala-192. P353L may alter the hydrogen bond of Arg-370 with the P-loop backbone by eliciting a conformational change, which is transmitted through helix G to \(\beta\)-sheet 8. Alternatively, Asp-348 hydrogen-bonds with N-1 of Arg-370, and the guanidinium group of Arg-370 hydrogen-bonds with the carbonyl of Ala-192. Ala-192 is a critical amino acid in the P-loop and forms numerous hydrogen bonds with the nucleotide (3, 12). Thus, P353L may suppress V194M by eliciting a conformational change that alters the hydrogen bond of Asp-348 with Arg-370. This conformational change in turn alters the hydrogen bond of Arg-370 with Ala-192 of the P-loop, thereby altering the conformation of the P-loop.

The hydrogen-bonding pattern of Asp-348 with Arg-370 and of Arg-370 with Ala-192 is not present in the \(\beta\text{E}\) conformation (Fig. 3B). In the \(\beta\text{TP}\) and \(\beta\text{EP}\) conformations, Arg-370 is 3.0 Å from the carbonyl oxygen of Ala-192, while this distance increases to 4 Å in the \(\beta\text{E}\) conformation. This shift of 1 Å is sufficient to break the hydrogen bond between Arg-370 and Ala-192. More dramatically, Asp-315 is 12.6 Å from Arg-370 in the \(\beta\text{E}\) conformation, while they hydrogen-bond in the \(\beta\text{TP}\) conformation. Thus, these interactions are specific for the conformations with nucleotide bound to the active site and may be important for stabilizing the \(\beta\text{TP}\) and \(\beta\text{EP}\) conformations. This hypothesis is supported by the fact that Pro-353 is in a catch region that interacts with the \(\gamma\)-subunit in the \(\beta\text{E}\) conformation (3). Furthermore, Pro-353, Arg-370, and Asp-348 are conserved in all 56 sequences of F-type ATPases in the SWISS-PROT database,\(^3\) suggesting that these residues are critical.

Mutagenesis of the residue that corresponds to Asp-348 in E. coli to Val (D301V) resulted in an enzyme that was defective in assembly of the ATPase complex (34). Therefore, this residue is important, minimally, for forming a stable structure of the ATPase. There have been no reports on mutagenesis of residues corresponding to Pro-353 or Arg-370, but it is of interest to determine their roles in the structure and function of the ATPase. The identification of a suppression mutation in the catch region that suppresses a mutation in the P-loop demonstrates genetically that the two regions are conformationally coupled. Further studies are required to determine conclusively if Pro-353, Arg-370, and Asp-348 are critical in the conformational coupling cascade during the catalytic cycle.

Interaction Pathways for T237I—The crystal structure indicates that Thr-237 interacts with Lys-209 in helix B (Fig. 4A). Thr-237 is 20 Å from the \(\beta\)-phosphate of bound ADP, so again, the mechanism of suppression must be via long-range interactions. Suppressor T237I is located within helix C of the nucleotide-binding domain. Helix B occurs just after the P-loop, and Val-198 actually is at the beginning of helix B. This suggests that suppressor T237I interacts with Lys-209, and this in turn
changes the conformation of the P-loop to suppress the initial V194Y mutation. However, this interaction is not present in the βp conformation (Fig. 4B). Thr-237 is 3.8 Å from Lys-209 in the βp state, but 10.3 Å apart in the βe conformation. Thus, this interaction is important in the conformation that binds nucleotide, but not in the conformation that does not bind nucleotide.

Lys-209 is not a well conserved residue, but the size of the residue appears to be important (cf. Fig. 6). In E. coli, for example, the corresponding residue is Ile. This indicates that the charged group is not critical, but the steric interactions may be important. Replacement of Lys-209 with Val in yeast results in an enzyme that has a 3-fold increase in the K_m for ATP and GTP and a 3-fold decrease in the k_cat for ATP binding. However, there was no change in the V_max for ATP hydrolysis as compared with the wild-type enzyme. This single change to a residue that is similar to that found in the E. coli enzyme significantly modified the kinetics of the yeast enzyme. Possibly, Ile at this position in the E. coli enzyme can account for some of the biochemical differences observed between the E. coli and mitochondrial enzymes (35–37).

The interaction of Thr-237 with Lys-209 is proposed to be important for modulating the activity of the enzyme. The importance is supported by the following data. First, T237I suppresses a P-loop mutation and thus must be able to alter the conformation of the active site. Since the P-loop forms multiple hydrogen bonds with the nucleotide, changes in the conformation of the P-loop have the potential of altering the hydrogen-bonding network. Second, T237I in an otherwise wild-type background is defective at 18 and 37°C, but not at 30°C as compared with the wild-type strain (Fig. 2). This indicates that the T237I interaction in the wild-type background modifies,

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4 V. Bulygin, unpublished results.
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but does not eliminate, the activity of the enzyme. Finally, mutagenesis of Lys-209 to Val has significant effects on the biochemistry of the ATPase. The variations of the residues at these two positions are proposed to modify the activity of the enzyme for the needs of the organism.

Interaction Pathways for L390F—The crystal structure of bovine F1 indicates that Leu-390 is in β-sheet 9 of the nucleotide-binding domain (Fig. 5A). Leu-390 is 24 Å from the β-phosphate of bound ADP in βE, so again, the mechanism of suppression must be via long-range interactions. The crystal structure indicates that Leu-390 interacts with Ala-181, which is in β-sheet 3 of the nucleotide-binding domain, and β-sheet 3 is adjacent to the P-loop. The mechanism of suppression is suggested to occur through the interactions of L390F with Ala-181, which distorts β-sheet 3 and alters the structure of the P-loop. The importance of this interaction is supported by a number of results. The L390F mutation suppresses V198S and V198K at all three temperatures (Figs. 2 and 3), shows allelic specificity (Fig. 3), and is defective in a wild-type background (Fig. 2), and mutagenesis to either Gly or Ala gives a negative growth phenotype (Fig. 2). Thus, Leu-390 forms an important interaction with Ala-181 as judged by genetic data and by analysis of the crystal structure of the ATPase. This interaction apparently can modify the structure of the P-loop since L390F is able to suppress the P-loop mutations V198S and V198K. The interaction between Leu-390 and Ala-181 is present in the βE, βTP, and βG conformations despite the large conformational differences observed in this region on the enzyme (Fig. 5B). Therefore, this interaction does not appear to contribute to determining, but is important in forming, the βE, βTP, and βG conformations.

Fig. 6 indicates that residues corresponding to Ala-181 and Leu-390 in other species or enzymes may, in part, define biochemical differences between these enzymes. The putative interacting residues can be placed into two groups: the mitochondrial or bacterial and the blue-green algae or chloroplast ATPases. The exact identity of the corresponding residues is variable, but they are generally limited to hydrophobic interactions, such as Ile with Ala, Ala with Ile, or Glu with Met. For blue-green algae or chloroplast ATPases, the pair is limited to Arg (or Lys in one case) and Met. It is not clear how an Arg/Met pair could fit in the same space as the Ala/Ile pair in the structure of the bovine enzyme. However, Asp or Glu at position 161 is always coincident with Arg at position 181, and the carboxylate may form a salt bridge with the guanidinium group of Arg. These differences in primary sequence may provide unique properties to the chloroplast and blue-green algae enzymes (38, 39). Mutagenic and biochemical studies on these residues in the yeast or bacterial enzyme should provide insight into the function of this putative salt bridge.

Although suppressor mutations of mutants in the P-loop of the E. coli ATPase have been reported (40, 41), there are a number of important differences compared with this study. In the E. coli study, the initial mutations were in Gly-149 (Gly-190 in yeast). In the bovine enzyme, this residue has very unusual dihedral angles that only allow Gly at this position (3). Assuming that the geometry of the P-loop is the same for the E. coli enzyme, then the geometry of the P-loop must be altered in the mutations at this residue. Suppressors of these mutations may be limited to those that can have a broad effect on the formation of the P-loop. Furthermore, the primary structural constraints of the E. coli P-loop are different from those of yeast. Although there has not been an extensive examination of the P-loop constraints in E. coli, Ser can replace Gly-149, and mutant F1 has nearly normal activity (35, 36). This is in contrast to the yeast enzyme, where the corresponding mutation, G190S, severely impairs F1 activity (19). A third important difference is that the primary sequence of the β-subunit from E. coli is more divergent from bovine than is yeast. The biochemical differences between the E. coli and mitochondrial enzymes must certainly be defined by the differences in their primary structures. However, primary structural differences may also change possible replacements that could suppress any given mutation, such as mutations in the P-loop mutations. For these reasons, the suppressors identified in the E. coli enzyme need not correspond to those identified in yeast.

The suppressors of mutations in the P-loop in the E. coli studies were G172D, S174F, D192V, and V198A. These residues are located at the beginning of β-sheet 4, in β-sheet 4, in helix C, and at the end of helix C, respectively. Interestingly, these suppressors are clustered and are in the region of the yeast P-loop suppressor T237I. The clustering of these suppressor mutations provides additional evidence that helix C and
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Fig. 6. Corresponding residues from a number of species and enzymes (see Footnote 3) that are proposed to interact. The residues that correspond to Lys-209 and Thr-237 are shown in A, and residues that correspond to Ala-181, Leu-390, and Ser-161 are shown in B. Sequences were aligned using MaxHom (28) and the SWISS-PROT data base. The primary sequences of the β-subunit of the ATPase were ordered into mitochondrial, bacterial, blue-green algae (BG), and chloroplast sequences, as shown. There were only partial sequence data available for sequence 27, and the space indicated that the sequence is not known. The dot in sequence 34 (A) indicates that the MaxHom program did not align this residue with the database sequences.

β-sheet 4 affect the conformation of the P-loop and thus the biochemistry of the enzyme.

Although some residues may diverge because their importance is relatively minor in defining the biochemistry of an enzyme, there must be other residues that diverge due to the different requirements of the organism or organelle. Suppressor studies may be an important tool for the identification of residues that are important in determining the biochemistry of the enzyme. The suppressors identified in this study all originated from mutations that were in or near the P-loop of the mitochondrial ATPase. As such, they all are able to correct a defect located in this critical motif. Since two of the putative interacting pairs of residues are not strictly conserved between species and enzymes, Thr-237/Lys-209 and Ala-181/Leu-390, it is suggested that this divergence can be responsible, in part, for their biochemical differences. Of course, there are five different subunits that compose the F₁ ATPase, each with divergent residues. Any, or many, of these differences may contribute to the biochemical differences observed between enzymes. However, certainly not all of the residues that are divergent are important for modulating the kinetics of the enzyme, as is postulated for the interacting pairs Thr-237/Lys-209 and Ala-181/Leu-390.

The P-loop undergoes dramatic conformational changes in the β-loop or β₁ loop to the βα loop (Ref. 3 and Figs. 3-5) and possibly in the transition from the high to the low affinity site in the catalytic mechanism. The understanding of the conformational coupling pathway from the proton, to F₀, to F₁, to the catalytic site requires molecular details of essential residues that interact and trigger this transition. Suppressor studies may be an important tool to identify interacting residues, such as Asp-348, Arg-370, and Ala-192 in the P-loop, which provide evidence for this region being able to modify the structure of the P-loop. A hydrogen-bonding network between Asp-348, Arg-370, and Ala-192 in the P-loop is proposed to effect this coupling. Genetic and biochemical studies indicate that the proposed interactions are critical for the activity of the enzyme. Analysis of the primary sequences of the β-subunit of 56 different sequences of F-type ATPases indicates that the interacting residues are not strictly conserved. Therefore, amino acid variations at these positions may be determinants of their unique biochemical properties.

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