Escherichia coli ATP Synthase α Subunit Arg-376: The Catalytic Site Arginine Does Not Participate in the Hydrolysis/Synthesis Reaction but Is Required for Promotion to the Steady State†

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ABSTRACT: The three catalytic sites of the F_OF_1 ATP synthase interact through a cooperative mechanism that is required for the promotion of catalysis. Replacement of the conserved α subunit Arg-376 in the *Escherichia coli* F_1 catalytic site with Ala or Lys resulted in turnover rates of ATP hydrolysis that were 2×10^3-fold lower than that of the wild type. Mutant enzymes catalyzed hydrolysis at a single site with kinetics similar to that of the wild type; however, addition of excess ATP did not chase bound ATP, ADP, or Pi from the catalytic site, indicating that binding of ATP to the second and third sites failed to promote release of products from the first site. Direct monitoring of nucleotide binding in the αR376A and αR376K mutant F_1 by a tryptophan in place of βTyr-331 (Weber et al. (1993) *J. Biol. Chem. 268*, 20126-20133) showed that the catalytic sites of the mutant enzymes, like the wild type, have different affinities and therefore, are structurally asymmetric. These results indicate that αArg-376, which is close to the β- or γ-phosphate group of bound ADP or ATP, respectively, does not make a significant contribution to the catalytic reaction, but coordination of the arginine to nucleotide filling the low-affinity sites is essential for promotion of rotational catalysis to steady-state turnover.

The F_OF_1 ATP synthase complex utilizes a rotational mechanism to couple the disparate functions of H^+ transport and ATP synthesis (for reviews, see refs *1*−*4*). The membranous F_O sector has a subunit stoichiometry of $ab_2c_{\sim 12}$ and contains the transport domain. The soluble F_1 sector has five different subunits in a stoichiometry of $\alpha_3\beta_3\gamma\delta\epsilon$ and contains the catalytic domain. The minimal portion of the complex required for maximal ATP hydrolytic rates is made up of the homologous α and β subunits that alternate in a hexamer, and a single copy of the core γ subunit (*5*−*7*; for reviews, see refs *2* and *3*). This minimal complex is sufficient for carrying out rotational catalysis (*8*−*10*). The three catalytic sites are found primarily in each of the β subunits with some amino acids contributed from the neighboring α subunits (*7*). The rotation of the γ and *c* subunit complex is essential for the energy coupling between proton transport and enzyme catalysis (*11*).

At substoichiometric levels, ATP binds to the first catalytic site with extremely high affinity ($K_D \cong 10^{-10}$ M in *Escherichia coli*, refs *2*, *12*, and *13*). In unisite catalysis, ATP undergoes reversible hydrolysis and synthesis but products are released very slowly (k_{off} for Pi[1] $\sim 10^{-3}$ s^{-1} in *E. coli*, refs *14*−*17*). Product release is greatly enhanced by binding of ATP at lower affinity to the other two sites ($K_{D2} \cong 1$ μM, $K_{D3} \cong 10$ μM, ref *2*).

According to the binding change (*18*, *19*) and rotational catalytic mechanisms (see ref *3* for a review), ATPs bound to the second and third sites, in turn, become substrates in a cyclical fashion upon conformational changes driven by rotation of the γ subunit. Asymmetry of the complex and interactions among the three sites are essential features of the rotational mechanism. An $\alpha_3\beta_3$ complex without the rotor γ subunit lacks structural (*20*) and functional (*21*) asymmetry, and the three sites act identically and independently of each other (*22*). Mg^{2+} is also required to establish the three classes of sites (*23*).

In this study, we examined the role of the apparent negative cooperativity among catalytic sites in the promotion of steady-state catalysis. To do this, we took advantage of enzymes with replacements of αArg-376, an α subunit residue in the catalytic sites and directly involved in coordinating the phosphate chain (*7*). Soga et al. (*24*) previously reported that the αArg-376 to Cys mutant enzyme had a greatly reduced steady-state turnover 4×10^3-fold lower than that of the wild type, while the effects on unisite catalysis were relatively small. We have made additional replacements of αArg-376 and assessed these enzymes for nucleotide binding and utilization of binding energy to drive catalysis. The mutant enzymes are able to use binding energy to attain the optimal conformation for catalysis; however, binding of nucleotide to the second and third sites fails to

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[1] Abbreviations: AMPPNP, 5′-adenylylimidodiphosphate; Pi, inorganic phosphate.

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Table 1: Unisite and Multisite Catalytic Rate Constants Determined for Mutant and Wild-Type F_1[a]

F_1 preparation	multisite k_{cat} (s^{-1})	k_{+1} ($\times 10^5$ M^{-1} s^{-1})	k_{-1} ($\times 10^{-3}$ s^{-1})	$K_{D,ATP}$ ($\times 10^{-9}$ M)	k_{+2} (s^{-1})	k_{-2} (s^{-1})	k_{+3} ($\times 10^{-3}$ s^{-1})	k_{-3} ($\times 10^{-4}$ M^{-1} s^{-1})	k_{+4} ($\times 10^{-4}$ s^{-1})	k_{-4} ($\times 10^3$ M^{-1} s^{-1})
Arg-376 (wild type)	58	1.5	0.25	1.7	0.17	0.11	2.3	4.6	3.4	7.0
αLys-376	0.022	0.84	1.2	14	0.07	0.07	3.0	0.96	3.2	3.1
αAla-376	0.026	0.50	1.1	22	0.07	0.07	2.8	0.77	3.8	2.8

[a] Unisite catalytic rates were determined at 25 °C using 0.25 μM [γ-^{32}P]ATP and 0.5 μM F_1 as previously described (*12*, *14*−*16*). Values for k_{-3} were estimated as described by Al-Shawi and Senior (*16*).

activate product release from the first site. The asymmetry of the three sites is maintained, but communication between sites is blocked, resulting in a loss of the promotion of catalysis.

EXPERIMENTAL PROCEDURES

Materials. Oligonucleotides were synthesized by Sawady Technology Inc., Tokyo. Enzymes for DNA manipulation were from Takara (Kyoto, Japan) or New England Biolabs (Beverly, MA). Other reagents used were of the highest grade commercially available.

Plasmids. Plasmid pBWU18, which was derived from pBWU13 and carried all eight structural genes of the ATP synthase (*25*), was modified by site-directed mutagenesis to introduce silent mutations, creating restriction sites *Nar*I in *uncA* codon αGly-379 and *Eco*52I in codon αPro-366. Double-stranded DNA cassettes were synthesized corresponding to the sequence between the *Nar*I and *Eco*52I sites and contained replacements for αArg-376 (CGT) by Lys (AAA) or Ala (GCA). The cassettes were ligated into a subclone of *uncA* (α subunit) between the *Sph*I and *Csp*45I sites (*26*), sequenced to confirm the presence of the mutation, and then moved to pBWU18. βTyr-331 (TAC) was replaced by Trp (TGG) by polymerase chain reaction using a primer containing the mutation and the neighboring *Eco*52I restriction site (TCTCTGGGTATCTGGCCGGCCGTTGA; mutation underlined). The PCR product was ligated into a subclone of *unc*D (β subunit) from *Sac*I to the *Aor*51HI sites (*27*), the sequence confirmed, and then the PCR product moved to pBWU18.

Biochemical Procedures. Membrane vesicles were prepared from logarithmic phase cells grown on glycerol (*28*). F_1 was isolated and purified as previously described (*29*) and used to determine unisite and multisite catalytic parameters (*12*, *15*). Tryptophan fluorescence emission at 360 nm was measured in a Hitachi spectrofluorometer F-3000 using 295 nm excitation light. Nucleotides bound to F_1 were depleted by centrifuge columns (*30*). Maximal tryptophan fluorescence values were determined using βY331W F_1 that was completely depleted of nucleotide. K_D values for Mg ADP or Mg AMPPNP were estimated as described previously (*31*). Nucleotides were analyzed by HPLC using a CLC-ODS column (Shimadzu, Kyoto, Japan).

RESULTS

Catalytic Properties of Enzymes with αArg-376 Substitutions. To further test the role of the conserved αArg-376 in cooperativity, two mutations, Lys (αR376K) and Ala (αR376A), were introduced into the *unc* operon overexpression plasmid, pBWU18, and the complex was expressed in the *unc*-deleted strain, DK8 (*32*). Like αR376C (*24*), these mutant strains were unable to grow dependent upon oxidative phosphorylation with sodium succinate as a sole carbon source, and the membrane fraction contained very little ATP hydrolytic activity. For both mutants, the amount of ATP synthase protein in the membranes was not significantly different from that of the wild type (data not shown). The mutant F_1 complexes were purified by a procedure developed for the wild-type enzyme (*29*) and found to be stable.

In standard conditions with saturating ATP (4 mM ATP, 2 mM $MgCl_2$, and 20 mM Tris−HCl at pH 8.0), the steady-state ATP hydrolytic activities of purified F_1 complexes from αR376A and αR376K mutant strains were at least 2×10^3-fold lower than that of the wild type (Table 1), which was similar to that reported for the αR376C mutant (*24*). Varying the ATP/Mg^{2+} ratio, concentration of KCl, or pH of the assay mixture did not alter the relative activities, which may have been expected if the mutant enzyme complexes were destabilized (*33*). Moreover, removal of ADP using pyruvate kinase and phosphoenolpyruvate did not affect activity, indicating that the mutant enzymes were not in a Mg ADP inhibited state (data not shown; see ref *34*).

The effects of the amino acid replacements on the elementary steps of ATP binding, hydrolysis/synthesis, and Pi and ADP release were assessed in the high-affinity catalytic site by using substoichiometric concentrations of ATP. The unisite conditions allow the analysis of the catalytic site structure in the absence of site-to-site interactions (*12*, *14*−*17*). To prepare for unisite analysis, the wild-type and mutant enzymes were first treated by passage over two centrifuge columns (*30*). HPLC analysis showed that nucleotide was completely removed (data not shown). As seen in Table 1, the effects of the αArg-376 amino acid replacements, Ala and Lys, were not large compared to those of other mutations in the active site (see refs *27* and *35*). For ATP binding (k_{+1}), the mutant rate constants were 2−3-fold lower than that of wild type, and those for ATP release (k_{-1}) were about 5-fold higher, resulting in K_D values for ATP that were 8- or 13-fold lower than that of the wild type. The step of chemistry in the mutant enzymes was only slightly affected. Hydrolysis (k_{+2}: $F_1 < ATP \rightarrow F_1 < ADP{\cdot}Pi$) and synthesis ($k_{-2}$: $F_1 < ADP{\cdot}Pi \rightarrow F_1 < ATP$) rates were slightly slower, and the equilibrium constant, K_2, remained close to unity, indicating that the loss of the arginine did not substantially affect the ability of the unisite to catalyze the reaction. Pi binding (k_{-3}) was the most noticeably affected with a 6-fold decrease in rate.

We used the rate constants to calculate binding energy utilization. The use of this analysis was validated by the establishment of linear free energy relationships of these mutants with many others (*36*). We found that the αArg-376 replacements resulted in only minor effects. The differences in energy profiles between the mutant and wild-type enzymes shown in Figure 1 indicated that the unisite

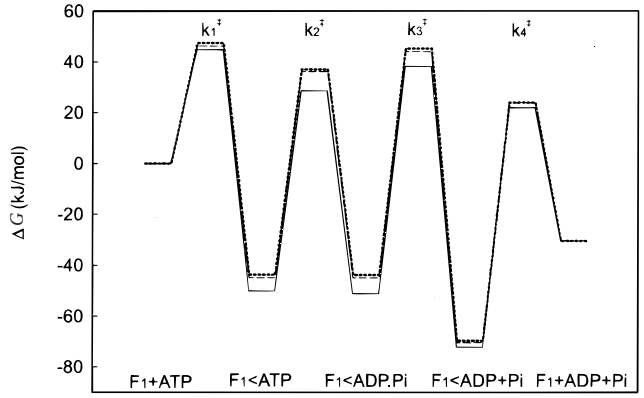

FIGURE 1: Gibbs free energy profiles for ATP hydrolysis by F_1 under unisite conditions for αR376A and αR376K enzymes. Energy levels are illustrated for wild-type *E. coli* F_1 (solid line), αR376A mutant F_1 (dashed line), and αR376K mutant F_1 (dotted line). The values were calculated as previously described (*16*, *47*).

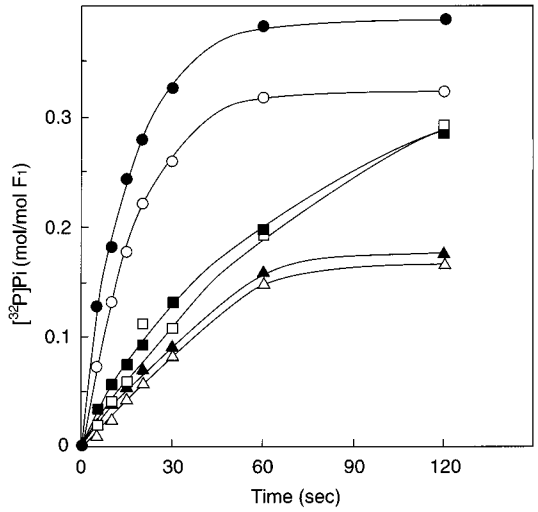

FIGURE 2: Acid quench and cold-chase experiments of αArg-376 mutant F_1. A 0.5 μM sample of wild-type F_1 (circles) or αR376K (squares) or αR376A (triangles) mutant F_1 was mixed with 0.25 μM [γ-^{32}P]ATP at 25 °C in a buffer containing 50 mM Tris−SO_4, 1 mM K_2HPO_4, 0.5 mM $MgSO_4$, pH 8.0. The reactions were stopped at the indicated times by addition of 0.2 M perchloric acid (acid quench, open symbols), or by addition of 4 mM ATP followed by a 10 s incubation and then by addition of 0.2 M perchloric acid (cold chase, closed symbols) (*15*).

had only minor perturbations in its ability to coordinate substrate in carrying out the step of chemistry and confirmed that αArg-376 does not participate in unisite catalysis.

Defective Promotion of Pi Release from αArg-376 Mutants. The relatively small effects on the unisite catalytic structure and the greatly reduced steady-state ATP hydrolysis activity by the mutant enzymes suggested that occupancy of the second and third sites did not promote activation of multisite catalysis. This was demonstrated by the lack of chase of the ^{32}P label by addition of excess cold ATP to enzyme preincubated with [γ-^{32}P]ATP in unisite conditions (Figure 2). The lower amount of [^{32}P]Pi produced in the mutants was due to the lower ATP affinity (Table 1). Upon addition of excess ATP to the wild-type enzyme, [γ-^{32}P]-ATP was hydrolyzed and ADP and [^{32}P]Pi were released with a minimum rate constant of 19 s^{-1} (Table 2). In the case of the mutant enzymes, there was no difference between

Table 2: Release Rates (s^{-1}) of Product ADP and Pi from Mutant and Wild-Type F_1 upon Addition of Excess ATP[a]

F_1 preparation	[^{32}P]Pi (s^{-1})	[^{3}H]ADP (s^{-1})
αArg-376 (wild type)	19.3[b]	19.3[b]
αLys-376	0.0040	0.0064
αAla-376	0.0028	0.0070

[a] A 0.5 μM sample of mutant or wild-type F_1 preparation was incubated with 0.25 μM [γ-^{32}P]ATP or [^{3}H]ADP at 25 °C for 10 s. After the preparation was passed through a centrifuge column (*30*), 4 mM cold ATP was added and incubated for various lengths of time. Dissociated label was separated by passage through a centrifuge column. The apparent release rates of [^{32}P]Pi and [^{3}H]ADP were estimated as previously described (*12*, *14*−*16*). [b] Because of the strong catalytic cooperativity of the wild-type enzyme and difficulty of accurately determining these rate constants, the wild-type values for Pi and ADP release presented here were estimated from the multisite rate (Table 1).

acid quench and cold-chase rates (Figure 2). When measured directly by the centrifuge column, the [^{32}P]Pi release rate in the presence of 4 mM cold ATP was not increased over the unisite Pi release rate, k_{+3} ($\sim 3 \times 10^{-3}$ s^{-1}; Table 2). Labeled ATP bound to the unisite was not directly displaced from the enzyme by the cold chase (data not shown); therefore, binding of ATP to the other sites did not promote hydrolysis of bound ATP nor the release of products ADP and Pi.

Effects of αArg-376 Replacements on Nucleotide Binding. We suspected that disruption of cooperative interactions between catalytic sites would be reflected by changes in nucleotide affinities and the negative cooperative behavior observed in the wild-type enzyme. To assess the effects of the αArg-376 replacements on nucleotide binding to each of the catalytic sites, the β331 tryptophan mutant (βY331W) was used to monitor nucleotide occupancy (*31*). βTrp-331 interacts with the adenine ring of ATP upon its binding to the catalytic site and results in quenching of the tryptophan fluorescence. Extensive analyses by Weber and Senior (*2*) have established that the quenching of the fluorescence intensity is linear with the filling of the sites by adenine nucleotide, and that the catalytic properties are similar to those of the wild type (*31*).

F_1 preparations were purified and depleted of nucleotide by twice passing through a centrifuge column (*30*). The titrations of Mg ADP onto βY331W, βY331W/αR376A, or βY331W/αR376K F_1 are shown in Figure 3. Adequate nonlinear regression fits could be obtained utilizing two binding components for both Mg AMPPNP and Mg ADP. Although the values for the βY331W enzyme were in good agreement with those previously reported (*31*), we report fits to three components to emphasize the differences in affinities of the three sites (Table 3). In the αR376A and αR376K mutants, the first K_D values for Mg AMPPNP and Mg ADP were extremely high affinity and could not be resolved (Table 3). Our result for Mg ADP binding to the αR376K enzyme was similar to that of Nadanaciva et al. (*37*). However, there is an apparent disagreement to the K_D for ATP determined by the kinetic experiments described in Table 1. In this case, the K_D was calculated to be approximately 10-fold *higher* than that of the wild type. The discrepancy is likely due to the presence of βY331W. In the presence of the βY331W residue, HPLC analysis found that 0.5−1.0 mol of nucleotide/mol of enzyme, wild-type and mutant, could not be removed (data not shown). This is in contrast to the αR376A and

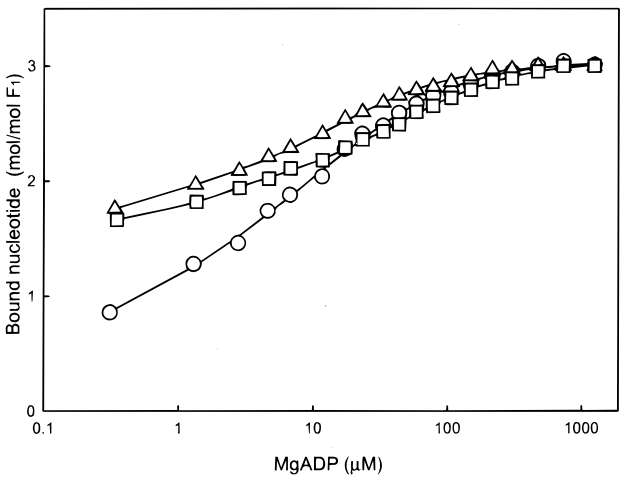

FIGURE 3: Filling of the catalytic sites of βY331W/αR376A and βY331W/αR376K F_1 with Mg ADP. Titration of Mg ADP onto αR376A (triangles) or αR376K (squares) mutant F_1 or wild-type αArg-376 F_1 (circles) was followed by monitoring the fluorescence of βY331W (excitation, 295 nm; emission, 360 nm). Prior to the nucleotide titration, enzymes were treated to remove nucleotide by passage over two centrifuge columns as described previously (*30*). The maximal fluorescence, or zero point for no nucleotide bound, was determined from the fluorescence of the wild-type enzyme completely devoid of nucleotide (as determined by HPLC analysis). Catalytic site occupancies were calculated from fluorescence quenching as previously described (*31*). Solid lines show the fit of the data by nonlinear regression analysis using a model for three binding sites (see Table 3).

Table 3: Effects of αArg-376 Mutations on AMPPNP and ADP Binding[a]

nucleotide	F_1 enzyme	$K_{D,1}$ (μM)	$K_{D,2}$ (μM)	$K_{D,3}$ (μM)
Mg AMPPNP	αArg-376 (wild type)	0.10	0.41	64
	αLys-376	NR[b]	8.4	384
	αAla-376	NR	NR	101
Mg ADP	αArg-376 (wild type)	0.098	3.6	26
	αLys-376	NR	0.55	47
	αAla-376	NR	0.23	16

[a] Nucleotide dissociation constants determined with the βY331W mutant F_1. The equation used for fitting is bound nucleotide = n[NUC]/{$K_{D,1}$ + [NUC]} + n[NUC]/{$K_{D,2}$ + [NUC]} + n[NUC]/{$K_{D,3}$ + [NUC]}, where [NUC] is the concentration of unbound Mg ADP or Mg AMPPNP. In all cases, the proportion of each K_D (n) was close to 1. [b] NR: Not resolved because the site was saturated at the lowest concentration of nucleotide added, indicating that the dissociation constant was lower than 0.05 μM.

αR376K mutant enzymes (without βY331W), which could be made completely devoid of nucleotide (see above). The occupied site is apparent in the fluorescence titrations which have approximately one site already filled (Figure 3). While the second and third K_D values varied from those of the wild type in different ways, similar trends were followed. Most importantly, it is apparent that the catalytic sites in the αR376A and αR376K mutant enzymes retain different affinities, and therefore the asymmetry of the three sites.

DISCUSSION

The thorough kinetic analysis of ATP hydrolysis in the unisite allowed us to assess the ability of the mutant enzymes to achieve an optimal conformation for catalysis. By comparing the free energy profiles for ATP hydrolysis based on the kinetic constants for αR376A and αR376K mutants to those for a number of mutant F_1 enzymes (*36*, *38*), we found that the utilization of binding energy was not significantly different from that of the wild type (Figure 1). Although the binding affinity for ATP of this site is lower by about 1 order of magnitude (Table 1), the high-affinity unisite can achieve a conformation similar to that of the wild type, as it properly coordinates ATP and carries out reversible hydrolysis/synthesis (*36*). The true defect of the mutant enzymes becomes apparent in conditions that normally promote steady-state turnover of the enzyme. In the case of the wild type, products ADP and Pi are released from the unisite in response to filling of the second and third catalytic sites (*31*, *39*). In contrast, products in the unisite of the αR376A and αR376K mutant enzymes are not released upon filling the other active sites with ATP (Figure 2). In fact, steady-state hydrolytic rates of the mutant enzymes in the presence of high ATP concentrations are similar to the unisite hydrolytic rates (compare Tables 1 and 2). These data indicate that the enzyme is unable to undergo the conformational changes that result in release of products from the site of chemistry or to rotate to the next step of catalysis.

The analyses of the effects of the αArg-376 substitutions help to define the negative cooperative interactions between the catalytic sites. Titration of the mutant catalytic sites monitored by the fluorescence quenching of βTrp-331 showed that, similar to the wild type, three nucleotide binding sites with different affinities were still observed (Table 3). Interestingly, all three sites in the βY331W/αR376A or βY331W/αR376K enzymes were somewhat higher than the enzyme with βY331W alone, while the unisite analysis found that the αArg-376 replacements caused a lower affinity for ATP. The reason for this discrepancy is not clear; however, it does not appear to be due to a nonlinear fluorescence response in filling of the catalytic sites. Numerous other catalytic site amino acid replacements have been analyzed in the same manner (2), which apparently did not affect the linearity of the fluorescence response. For the nucleotide titrations monitored by fluorescence quenching described here, the only important point is that the experiments clearly show that the inherent asymmetry observed in the X-ray crystallographic structure (*7*, *40*) is retained in the mutant enzymes. We conclude that replacement of αArg-376 only disrupts the communication between the catalytic sites and the ability of the sites to shift conformation upon filling of the low-affinity sites with ATP.

αArg-376 is close to the γ-phosphate of ATP in the β_{TP} site and to the β-phosphate of ADP in the β_{DP} site (*7*). As pointed out by Weber and Senior (*2*), αArg-376 has one of the largest positional differences between the ATP- and ADP-filled sites (*7*). The results reported here show that αArg-376 does not contribute to formation of the pentacovalent transition state as has been previously suggested (*2*, *7*, *37*) because the αR376A and αR376K mutant enzymes were able to attain the near optimal catalytic conformation in the unisite. Consistent with this conclusion, the mutant enzymes had no cold chase of tightly bound ATP from the unisite nor was ATP release (k-1) promoted upon binding of ATP to the second and third catalytic sites. For comparison, some catalytic site mutants which cannot achieve proper catalytic site structure, such as βR246C and βM209I, release unisite

ATP upon binding ATP to the second or third catalytic sites (*41*).

Arginine residues are frequently fixed rigidly into position because of the large hydrogen bond capacity of the guanidinium group (*42*). The rigid position of the arginine may be a critical property of this residue because lysine cannot replace its function. We propose that filling of the second and third catalytic sites with ATP causes a shift in position of αArg-376 and probably other residues near the catalytic sites, such as αSer-347, αGly-351, αSer-373, and αSer-375 (*43*, *44*), which initiates the conformational change that is transmitted to the unisite. The conformational change in the unisite, which is carrying out reversible hydrolysis/synthesis, accelerates the reaction and leads to release of Pi and ADP. αArg376 is the linchpin in the cooperative mechanism.

This role for αArg-376 is different from that previously suggested on the basis of interpretation of the bovine mitochondrial F_1 structural model (*7*). It was suggested that the position of the αArg-376 (αArg-373 in bovine mitochondrial numbering) guanidinium group could help stabilize the negative charge of the pentacovalent transition state. Apparently in agreement with this interpretation, Nadanaciva et al. (*37*) found that replacing αArg-376 with Cys and Gln affected fluoroaluminate binding, an indicator of the formation of the pentacovalent transition state. They also reported that αR376K could bind fluoroaluminate, which they believed was a partial functional replacement by the lysine amide. If αArg-376 is critical for forming the transition state, then replacing this amino acid should strongly affect the steps of chemistry in the unisite. However, k_{+2} and k_{-2} (Table 1) of αR376K and, in particular, αR376A are similar to those of the wild type, demonstrating that the enzyme is able to pass through the transition state. We propose that the αR376K mutant bound fluoroaluminate because this enzyme has good unisite activity and the catalytic site can achieve the proper structure to carry out catalysis. Clearly in this case, determination of the kinetic constants was essential to understand the role of this catalytic site residue.

Because the release of Pi is essentially irreversible in the absence of a $\Delta\mu_{H^+}$, this step and the change of affinity for ATP are the major energy-utilizing steps of the enzyme cycle (*36*, *45*). We have previously proposed that the major conformational change in the catalytic sites occurs just prior to Pi release and that this change involves the rotation of the γ subunit (*3*, *45*). The effects of the αArg-376 replacements are consistent with this model. Filling of the second and third catalytic sites with ATP, which is required for maximal turnover (*31*), forces the positional shift of αArg-376 and triggers the rotation of the γ subunit, which is already poised in a high-energy state (*45*, *46*), leading to the change of conformation of all three catalytic sites. Without the arginine, the rotation cannot be triggered, and the rotation does not occur.

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