Journal of Bioenergetics and Biomembranes, Vol. 20, No. 4, 1988

MINI-REVIEW

Mechanism of F_1-ATPase Studied by the Genetic Approach[1]

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Received November 5, 1987

Abstract

E. coli F_1-ATPase has been studied mainly by the genetic approach. Mutations in either the α or β subunit modified the kinetics of multisite and uni-site hydrolysis of ATP. The mechanism of F_1-ATPase and the essential amino acid residues of β subunits are discussed.

Key Words: F_1-ATPase; H^+-ATPase; ATP synthase; *unc* operon; uni-site catalysis; ATP binding site; aurovertin.

Introduction

The H^+-ATPase (ATP synthase) of *Escherichia coli* is a complex membrane protein similar in composition and structure of its subunits and catalytic mechanism to the ATP synthases in mammalian mitochondria and plant chloroplasts. The catalytic entity F_1 (or F_1-ATPase) consists of five subunits α, β, γ, δ, and ε, and acts as an ATPase when it is purified. The proton pathway F_0 is the intrinsic membrane protein, and F_0 of *E. coli* has three subunits *a*, *b*, and *c*. Studies on the *E. coli* enzyme coded by the *unc* operon are advantageous, as genetic manipulation of this organism is easier than that of eukaryotic cells and the entire F_0F_1 and the subunits can be isolated. Analysis of defective mutant enzymes may eventually give pertinent information on the mechanism of ATP synthesis and hydrolysis. Amino acid replacements in mutant enzymes can be determined rapidly because the DNA

[1] Abbreviations used: P_i, inorganic phosphate; DCCD, dicyclohexylcarbodiimide.

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0145-479X/88/0800-0469$06.00/0

sequence of the *unc* operon is known. Applications of site-directed mutagenesis can be used to evaluate the roles of residues modified genetically or with protein chemical reagents and reactive nucleotide analogues. Thus the catalytic roles of specific residues can be established by genetic studies.

This short article summarizes recent studies by genetic approaches on the mechanism of ATP hydrolysis by *E. coli* F_1-ATPase. This is not intended to be a comprehensive review, but includes a critical discussion of the present situation with indications of the problems that should be studied further. For fields that are not covered here, readers should refer to other articles of this mini-review series and recent review articles (Futai and Kanazawa, 1983; Walker *et al.*, 1984; Senior, 1985). We have cited review articles rather than original papers as far as possible.

unc Operon, Its Mutants and Site-Directed Mutagenesis

unc Operon and Its Mutants

The *unc* operon coding for *E. coli* H^+-ATPase was cloned and its entire DNA sequence was determined. The order of the structural genes is *uncB* (*a*), *uncE* (*c*), *uncF* (*b*), *uncH* (δ), *uncA* (α), *uncG* (γ), *uncD* (β), and *uncC* (ε) (Kanazawa and Futai, 1982; Futai and Kanazawa, 1983). Operons coding for F_1 or F_0F_1 from *Rhodopseudomonas blastica* (Tybulewicz *et al.*, 1984), *Rhodospirillum rubrum* (Falk *et al.*, 1985), and a cyanobacterium *Synechococcus* 6301 (Cozens and Walker, 1987) and a thermophilic bacterium PS3 (Kagawa *et al.*, 1985) were sequenced. Genes coding for the eukaryotic enzymes were sequenced, mainly from mitochondrial and chloroplast genomes (Walker *et al.*, 1984). The primary sequences of bovine F_1 subunits were determined by protein sequencing (Runswick and Walker, 1983). The human β subunit was also determined from *c*DNA (Ohta *et al.*, 1988). The α and β subunits have highly conserved primary sequences, consistent with the presence of an active site in the β subunit or at the interface between the α and β subunits (Futai and Kanazawa, 1983; Walker *et al.*, 1984). Furthermore, α subunits are homologous to β subunits: 37% of the amino acid residues in the *E. coli* β subunit are homologous (identical or equivalent) to those in the *E. coli* α subunit.

E. coli mutants defective in H^+-ATPase were identified as strains unable to grow by oxidative phosphorylation of nonfermentable carbon sources such as succinate (Downie *et al.*, 1979). Mapping and cloning the mutant alleles became easier using a series of recombinant plasmids that carried different segments of DNA of the *unc* operon. A mutation in H^+-ATPase can be identified and mapped in a defined region of the *unc* operon or cistron,

when the mutant strain becomes capable of growing on succinate after recombination with a specific DNA fragment of the operon carried by a certain plasmid (Kanazawa *et al.*, 1983). The alleles of mutants can be cloned from their chromosomal DNA using a similar recombination assay, and mutational amino acid replacements can be identified from the change of the genetic codon (Noumi *et al.*, 1984b). Thus we can study the mechanism of F_1-ATPase or H^+-ATPase by analyzing mutant enzymes with defined amino acid substitutions. Many mutants of H^+-ATPase have been isolated in various laboratories, since Gibson and coworkers isolated one of the first *unc* mutants (Butlin *et al.*, 1971). So far, we have identified 168 independent mutants and have mapped 18% of them on the α subunit and 33% of them on the β subunit. This large number of mutants of the α and β subunits is consistent with the importance of these subunits for catalysis. Of these mutants, about 80% were defective in assembly, and mutant F_1's with altered subunit compositions could not bind to F_0, or were difficult to solubilize from membranes by the procedure used for the wild-type enzyme. These assembly mutants may be important for understanding subunit–subunit interaction(s) and the higher-order structure of the complex. Naturally we can study the defective catalytic properties of mutant F_1 only when it can be solubilized and purified. Mutant F_1's often become unstable after solubilization and are not easy to purify, so special precautions are necessary for each mutant F_1.

Application of Site-Directed Mutagenesis to the β Subunit

Site-directed mutagenesis has been useful for studying the roles of amino acid residues replaced in mutants or those modified with protein chemical reagents and reactive nucleotide analogues. Two experimental systems for this purpose have recently been developed. Noumi *et al.* (1987c) introduced oligonucleotide-directed mutations on a cloned *uncD* gene carried by a plasmid, and introduced the mutant plasmids into a deletion strain of F_1F_0 (Δ*uncB–C*). Excess β subunit was synthesized in the deletion mutants and could be purified to apparent homogeneity from the cytoplasmic fraction. The catalytic and assembly properties of the isolated β subunit could be studied, as the $\alpha\beta\gamma$ complex with ATPase activity could be reconstituted from a combination of the isolated α, β, and γ subunits of the wild-type. The advantage of this experimental system is that the properties of the β subunit could be studied, even when it was defective in forming intact F_1. Needless to say, the possibility of additional mutation(s) other than that intended should be excluded by a genetic procedure or DNA sequencing. A similar experimental system was established for introducing mutations into the thermophilic β subunit (Ohtsubo *et al.*, 1987a).

Parsonage *et al.* (1987b) developed a different experimental system. They cloned a fragment carrying the *uncD* and *uncC* genes and introduced a

mutation in the *uncD* gene carried by the resulting recombinant plasmid pDP31. The mutant and wild-type pDP31 were used to transform a polar mutant strain (induced by Mu-phage) in which the *uncD* and *uncC* genes were not expressed. The β and ε subunits synthesized, depending on the plasmid, could assemble and form wild-type or mutant F_1 with other subunits synthesized from chromosomal genes. The advantage of their experimental system is that mutant F_1 can be purified easily from the membranes by established procedures.

Catalytic Mechanism of F_1-ATPase

Uni-site and Multisite Hydrolyses of ATP

Purified F_1 still retains ATPase activity, and its two types of kinetic mechanisms (uni-site and multisite hydrolysis of ATP) were demonstrated with bovine heart F_1 (Grubmeyer *et al.*, 1982) and confirmed with *E. coli* F_1 (Duncan and Senior, 1985; Noumi *et al.*, 1986a). When the concentration of ATP is lower than that of F_1, ATP binds to a single catalytic site and is hydrolyzed only slowly (uni-site hydrolysis). The equilibrium constant of $F_1 \cdot ATP \rightleftharpoons F_1 \cdot ADP \cdot P_i$ is 0.5–1.0, suggesting that $F_1 \cdot ATP$ can be formed from $F_1 \cdot ADP \cdot P_i$ without energy input. On the other hand, in the presence of excess ATP under steady-state assay conditions, ATP binds to all three catalytic sites and P_i is released at the maximal velocity (multisite hydrolysis). The rates of P_i release by multisite hydrolysis (k_7) by bovine and *E. coli* F_1 are 10^6 and 10^{4-5} times, respectively, higher than those by uni-site hydrolysis (k_3). The higher rate of multisite hydrolysis clearly indicates positive cooperativity between different catalytic sites. Boyer and his colleagues (Choate *et al.*, 1979; Hutton and Boyer, 1979) showed that bovine F_1 catalyzed an exchange between water oxygen and oxygen of phosphate during hydrolysis of ATP at low concentration, but they did not observe this exchange at a high ATP concentration. These findings suggest that ATP hydrolysis at the catalytic site of F_1 is reversible, although the reversibility becomes lower at higher ATP concentration because of faster cooperative hydrolysis of ATP, indicating that cooperative interactions can occur between different catalytic sites. Wood *et al.* (1987) recently observed similar P_i–water exchange during ATP hydrolysis by *E. coli* F_1.

Inhibition of Multisite Hydrolysis of ATP

Catalytic cooperativity requires transmission of conformational changes between multiple catalytic sites. Thus there are inhibitors of only multisite catalysis that bind to the region required for transmission of conformational

change. Similarly, mutations may differentiate the two kinetic mechanisms of ATP hydrolysis. Addition of 200 μM NaN_3 inhibited multisite ATPase activity of F_1 noncompetitively with a K_i value of 7 μM and caused 93% inhibition at 25° C (Noumi *et al.*, 1987b), but it did not affect the initial rate of uni-site hydrolysis (measured as P_i bound to F_1 and released into the medium). In the presence of the same concentration of azide, F_1 showed a slightly lower k_1 (rate of ATP binding to F_1), whereas the values for k_{-1} (rate of release of ATP from F_1) and k_3 (rate of release of P_i from F_1) were essentially unchanged (Table I). Azide slightly changed the ratio of $F_1 \cdot ATP$ to $F_1 \cdot ADP \cdot P_i$: the ratio was about 3:2 in the presence of azide and 2:1 in its absence. Thus azide did not inhibit uni-site hydrolysis, although it caused slight change in the kinetics. Therefore it inhibited multisite hydrolysis mainly by lowering the catalytic cooperativity. Mutations in both α and β subunits changed the sensitivity to azide: the residual multisite activities of KF43 defective in the β subunit (Arg-246 → His) (Noumi *et al.*, 1986a) and *uncA401* defective in the α subunit (Senior *et al.*, 1984) were insensitive to azide.

Tommasino and Capaldi (1985) suggested that DCCD also blocks cooperativity between catalytic sites, although this compound affects catalytic sites. They found that 95% inhibition of multisite ATPase activity required incorporation of 1 mol of DCCD, whereas 75% inhibition of the initial rate of uni-site hydrolysis was observed only after incorporation of 2 mol DCCD/mol of F_1. DCCD binds to Glu-192 of the β subunit under conditions for inhibition of multisite activity (Yoshida *et al.*, 1982). Thus the Glu-192 residue or the region in its vicinity may be important for catalytic cooperativity. Consistent with this finding, the importance of Glu-185 near Glu-192 of the β subunit in subunit–subunit interaction was suggested from mutant studies (Noumi *et al.*, 1986b) and site-directed mutagenesis (Noumi *et al.*, 1987c): on replacement of Glu-185 by Lys or Gln, the β subunit was unable to form a $\alpha\beta\gamma$ complex.

Loss of catalytic cooperativity may be due to a defect in transmission of conformational changes between different subunits. The effect of azide on transmission of conformational changes was studied using aurovertin as a fluorescent probe (Noumi *et al.*, 1987b). This compound is known to bind to the β subunit. F_1 had similar K_d values and maximal fluorescence for aurovertin with or without azide, suggesting that azide had no effect on the aurovertin site. However, azide lowered the fluorescence of aurovertin in the presence of Mg^{2+}. The fluorescence of the aurovertin bound to F_1 was enhanced by addition of ADP following two kinetic phases (rapid and slow phases). The enhanced fluorescence was quenched with Mg^{2+} and further with azide. Azide also changed the fluorescence response of the enzyme to ATP. Thus azide may affect transmission of conformational changes between the ligand site and aurovertin site in the β subunit.

Table I. Catalytic Properties of F_1-ATPase from Wild-Type and Mutant *Escherichia coli*

	Wild-Type			Mutant				
Strains	KY7485	KY7485	KY7485	KF43 (*uncD43*)	KF87 (*uncD87*)	*uncD484*[f]	*uncD412*[f]	*uncD431*[g]
Amino acid substitution	None	None	None	Arg-246 → His	Ala-151 → Val	Met-209 → Ile	Gly-142 → Ser	Arg-246 → Cys
Addition in assay	None	NaN_3[c]	AP_3-PL[d]	None	None	None	None	None
Uni-site hydrolysis[a]								
$k_1(M^{-1}s^{-1})$	8.0×10^4	5.6×10^2	8.0×10^2	5.3×10^4	$\geqslant 44 \times 10^4$	0.9×10^4	3×10^4	0.5×10^4
$k_{-1}(s^{-1})$	$\leqslant 6.0 \times 10^{-5}$	$\leqslant 6.0 \times 10^{-5}$	$\leqslant 12 \times 10^{-5}$	ND	ND	8×10^{-4}	3×10^{-4}	1.8×10^{-3}
$k_3(s^{-1})$	1.8×10^{-3}	1.4×10^{-3}	ND	3×10^{-2}	$\geqslant 0.2$	$\geqslant 5 \times 10^{-2}$	1×10^{-3}	$> 5 \times 10^{-2}$
Multisite hydrolysis								
$k_7(s^{-1})$[b]	78	6.2	1.6	1.9	4.7	0.22	12	1
Promotion of catalysis								
k_7/k_3	4.3×10^4	4.4×10^3	—	63	$\geqslant 24$	$\geqslant 5$	1×10^4	< 20
Reference[e]	1, 2	2	3	1	4	5, 6	5, 6	6

[a]Values of k_1, k_{-1}, k_3 are rate constants of uni-site catalysis:

$$F_1 \underset{ATP}{\overset{ATP\ k_1}{\rightleftharpoons}} F_1 \cdot ATP \underset{k_{-2}}{\overset{k_2}{\rightleftharpoons}} F_1 \cdot ADP \cdot P_i \underset{k_{-3}\ P_i}{\overset{k_3\ P_i}{\rightleftharpoons}} F_1 \cdot ADP \underset{ADP}{\overset{ADP}{\rightleftharpoons}} F_1$$

[b]Rate of hydrolysis of ATP in multisite catalysis (steady-state assay conditions).
[c]Uni-site and multisite ATP hydrolysis were assayed in the presence of 200 μM NaN_3.
[d]Uni-site and multisite ATP hydrolysis were assayed with wild-type F_1 with AP_3-PL (adenosine triphosphopyridoxal) bound covalently (about 1 mol/mol).
[e]Cited from Noumi *et al.*, 1986b (1), 1987b (2), 1987c (3); Hsu *et al.*, 1987 (4); Duncan and Senior, 1985 (5); and Parsonage *et al.*, 1987a (6).
[f]Cited from Duncan and Senior, 1985. Their wild-type rates were slightly different from ours: k_1, k_{-1}, k_3, and k_7 were 8×10^4, $< 4 \times 10^{-5}$, 1×10^{-3}, and 58, respectively. Thus k_7/k_3 from their assay was 6×10^4. We think the difference is due to the slightly different assay conditions between the two laboratories.
[g]Cited from Parsonage *et al.* (1987a).

Unlike results with azide or DCCD, F_1 modified with about 1 mol of adenosine triphosphopyridoxal showed essentially no uni-site or multisite hydrolysis of ATP (Noumi *et al.*, 1987a) (Table I). The rate of binding of the modified F_1 with ATP decreased to 10^{-2} of that of F_1. Thus adenosine triphosphopyridoxal bound to an active site and completely abolished catalysis by the two remaining sites, consistent with the idea of cooperative interactions of three catalytic sites.

Alteration of Catalysis Due to Mutations in α and β Subunits

Defective Multisite Catalysis and Conformational Transmission of Mutant F_1's. As mentioned above, mutations may differentiate the two kinetic mechanisms. Furthermore, in contrast to studies with inhibitors, mutations can define changes of the enzyme at the level of amino acid residues. Single amino acid substitutions in either the α or β subunit resulted in loss of multisite hydrolysis: F_1-ATPases from mutants *uncA401* (Ser-373 → Phe in α subunit) (Noumi *et al.*, 1984a), *uncA447* (Gly-351 → Asp in α subunit) (Maggio *et al.*, 1987), *uncA453* (Ser-375 → Phe in α subunit) (Maggio *et al.*, 1987), KF43 (Arg-246 → His in β subunit) (Noumi *et al.*, 1986b), KF87 (Ala-151 → Val in β subunit) (Hsu *et al.*, 1987), *uncD484* (Met-209 → Ile in β subunit), and *uncD412* (Gly-142 → Ser in β subunit) (Parsonage *et al.*, 1987a; Duncan and Senior, 1985) had low multisite ATPase activities. Defective conformational transmission between ligand binding sites was studied in detail with F_1's from strains *uncA401* and KF87. The fluorescence of aurovertin bound to wild-type β was decreased by Mg^{2+} and further by ATP, whereas that of aurovertin bound to *uncA401* F_1 was increased by Mg^{2+} (Wise *et al.*, 1981; Kanazawa and Futai, 1982). Similarly, the fluorescence of the wild-type was decreased by P_i, but that of the mutant was increased by P_i (Kanazawa *et al.*, 1984). The fluorescence of aurovertin bound to KF87 was not affected by Mg^{2+}, ATP, or ADP, although the mutant and wild-type F_1's had similar high-affinity K_d's for aurovertin (Hsu *et al.*, 1987). The fluorescence of the ATP analogue (2′-(5-dimethylamino-naphthalene-1-sulfonyl)-amino-2′-deoxy ATP) bound to wild-type F_1 was increased appreciably by P_i, while the same fluorescence in the *uncA401* F_1 was not affected by P_i (Kanazawa *et al.*, 1984). Therefore mutations in either the α or β subunit showed defective conformational transmission between ligand-binding sites, and this defect lowered catalytic cooperativity.

An interesting problem was whether F_1 in which one β subunit was defective in conformational transmission was still active in multisite catalysis. This question was examined by analyzing hybrid F_1's constructed from the β subunits of the wild-type and an appropriate mutant. A complex with ATPase activity could be reconstituted from the α, β, and γ subunits of the

wild-type. Theoretical analysis of reconstitution experiments using β subunits from the wild-type and KF43 (Arg-246 → His) suggested that hybrid enzymes carrying both the mutant and wild-type β subunit were inactive in multisite hydrolysis (Noumi *et al.*, 1986b), supporting the notion that three intact β subunits are required for multisite hydrolysis of ATP.

Altered Uni-site Catalysis in Mutant F_1's. It is of interest to study the kinetics of uni-site hydrolysis of F_1's from mutants defective in multisite hydrolysis. The kinetic parameters of uni-site hydrolysis of the mutant F_1's so far reported are summarized in Table I. The F_1 from *uncD412* (Gly-142 → Ser) had essentially the same values of k_1 (rate of binding of ATP) and k_3 (rate of release of P_i) as the wild-type enzyme, although its k_{-1} (rate of release of ATP) was about 10-fold higher (Duncan and Senior, 1985). Other mutant F_1' showed different values of k_1 and k_3 from those of wild-type F_1: the F_1's from *uncD484* (Met-209 → Ile) (Duncan and Senior, 1985) and KF43 (Arg-246 → His) (Noumi *et al.*, 1986b) had at least 10-fold higher k_3 values than that of the wild-type, and F_1 of KF87 (Ala-151 → Val) (Hsu *et al.*, 1987) had a 50- to 100-fold higher k_3 value. Similarly, the values of k_1 varied in different strains: the k_1 value of F_1 of *uncD484* (Duncan and Senior, 1985) was one-tenth that of the wild-type, that of KF43 was about the same as that of the wild-type (Noumi *et al.*, 1986), and that of KF87 was more than 5-fold higher (Hsu *et al.*, 1987). The properties of KF43 F_1 are similar to those of (Arg-246 → Cys) mutants (*uncD431*, Parsonage *et al.* (1987a) or KF104, Kuki, Noumi, Maeda, and Futai, unpublished results) in which the same residue as in KF43 is affected. Substitution of Cys at position 246 seemed more harmful for cooperativity than substitution of His, and the enzyme with the Cys substitution had lower multisite ATPase activity than that with the His substitution. The promotions of catalysis (ratio of multisite to uni-site rate, k_7/k_3) were 63 and $\leqslant$ 20-fold for the KF43 (Arg-246 → His) and *uncD431* (Arg-246 → Cys) enzymes, respectively. These results clearly indicate that the amino acid residues Gly-142, Ala-151, Met-209, and Arg-246 of the β subunit are closely related to the catalytic site of the β subunit.

It is noteworthy that both the uni-site and multisite kinetics of the F_1's from these mutants were altered by a single amino acid substitution, suggesting that uni-site and multisite ATP hydrolyses are closely related. These findings also support the assumption that uni-site and multisite hydrolyses are carried out by the same catalytic site.

Essential Amino Acid Residues for Catalysis by F_1-ATPase

Replacements of Glu-41, Glu-185, Gly-223, and Ser-292 of the β subunit by Lys, Lys, Asp, and Phe, respectively, resulted in defective assembly of the

F_1 complex (Noumi *et al.*, 1986a). As described above, Gly-142, Ala-151, Met-209, and Arg-246 of the β subunit and Ser-373 of the α subunit are essential for multisite and uni-site hydrolyses. Furthermore, Ser-174 of the β subunit may be important in Mg^{2+} binding, since replacement of this residue by Phe resulted in an altered divalent cation requirement for multisite ATPase activity (Kanazawa *et al.*, 1980; Noumi *et al.*, 1984b). These amino acid residues are conserved in all the β subunits so far reported except Gly-223 which is conserved in the subunits of mitochondria and bacteria (Futai and Kanazawa, 1983; Walker *et al.*, 1984). So far, nine independent β subunit mutants with defective catalysis have been identified and, interestingly, three of the mutations were of Arg-246 and three were of Ser-174 (Kuki, Noumi, Maeda, and Futai, unpublished results; Noumi *et al.*, 1986a; Parsonage *et al.*, 1987a). These results suggest that the numbers of essential residues in the β subunit are limited and that the possibility of isolating clones with other mutations is low.

Chemical modification experiments indicated more than 13 "*essential*" (or "important") amino acid residues in the β subunit. However, no mutants with mutations of these residues have been isolated so far, although more than 10 mutants have been identified so far, and some of them have been isolated independently at least twice, as discussed above. Thus, it becomes of interest to change "*essential*" residues by site-directed mutagenesis and study the properties of the resulting enzymes.

4-Chloro-7-nitrobenzofurazan reacts specifically with the β subunit at the residue equivalent to Tyr-297 of *E. coli* β (Andrews *et al.*, 1984a,b) and this residue is conserved in all the β subunits so far sequenced (Walker *et al.*, 1984). However, Parsonage *et al.* replaced this Tyr by Phe and found that 70% of the activity remained after the replacement (Parsonage *et al.*, 1987b). Essentially the same results were obtained using thermophilic F_1 (Ohtsubo *et al.*, 1987b). Similarly, Tyr-354 (site of the nucleotide affinity agent *p*-sulfonylbenzoyl-5′-adenosine) (Esch and Allison, 1978) was replaced by Phe, and this replacement had no effect on the F_1-ATPase activity (Parsonage *et al.*, 1987b). These results suggest that Tyr-297 and Tyr-354 do not have catalytic roles in F_1. Thus care is required in analyzing the mechanism of F_1 using these reagents, if they do not bind to catalytic sites. However, it is possible that these compounds to bind to the catalytic site, and they form a covalent bond with a residue in or around the catalytic site.

Glu-181 (Yoshida *et al.*, 1981) and Glu-192 (Yoshida *et al.*, 1982) correspond to the binding site of DCCD in thermophilic bacterial and *E. coli* F_1's, respectively. Recently Ohtsubo *et al.* (1987b) substituted Gln or Asp for Glu residues of the thermophilic β subunit corresponding to Glu-181 and Glu-192 of the *E. coli* subunit. They found that the $\alpha\beta\gamma$ complex could be formed from the mutant β subunit and the α and γ subunits from the

thermophilic bacterium, but that these complexes did not have ATPase activity. Therefore, the two Glu residues may be essential for the catalysis. These results are consistent with the predominant inhibitory effect of DCCD on multisite hydrolysis.

Since a portion of the sequence of the β subunit was first found to be similar to that of adenylate kinase (Kanazawa *et al.*, 1982), the sequence Gly-X-X-X-X-Gly-Lys-Thr (Ser) has been found in more than 40 nucleotide-binding proteins (Walker *et al.*, 1982; Fry *et al.*, 1985; Moller and Amons, 1985). In adenylate kinase this region forms a flexible loop that interacts with the α or γ phosphoryl groups of ATP. As discussed above, replacement of Ala-151 (the second residue downstream to Gly in the sequence) by Val lowered the rate of multisite hydrolysis and changed the kinetic parameters of the uni-site hydrolysis (Hsu *et al.*, 1987). It is interesting that the normal and activated oncogenic p 21 *ras* proteins have Gly and Val residues, respectively, at the corresponding positions of the homologous sequence (Tabin *et al.*, 1982; Reddy *et al.*, 1982). The replacement of Gly or Ala by Val may change the structure of the ATP binding site, possibly the orientation of Lys-16 (p 21 *ras* protein) or Lys-155 (β subunit). More directly, Parsonage *et al.* (1987b), replaced Lys-155 by Gln or Glu by site-directed mutagenesis. The membrane F_1-ATPase activities of the Gln and Glu-185 enzymes were 11 and 16%, respectively, of that of the wild-type. The mutant membranes had lower activity in ATP-driven pH gradient formation. These findings suggest that Lys-155 is essential for catalysis in F_1-ATPase. However, the results do not support the idea that the amino group of Lys-155 of β interacts with the phosphoryl group of ATP, because the Lys-155 $\rightarrow$ Gln mutation had a more severe effect on the enzyme than the Lys-155 $\rightarrow$ Glu mutation. They also introduced a Gly-154 $\rightarrow$ Ile mutation and found that it resulted in strong impairment of multisite catalysis, whereas a Gly-149 $\rightarrow$ Ile mutation did not affect this activity. Kagawa and coworkers replaced Lys-164 of the thermophilic β subunit (corresponding to *E. coli* Lys-155) by Ile and found that the mutant did not show multisite ATPase activity (Yohda *et al.*, 1987). These results suggest that this region is essential in multisite catalysis.

Conclusion

We have discussed studies by the genetic approach for understanding this complicated enzyme. One of the most important contributions of this line of approach has been the demonstration that the kinetics of uni-site and multisite hydrolyses could be modified by a mutation: replacement of a single amino acid in either the α or β subunit could result in loss of multisite catalysis. Further analysis of such mutations may indicate the molecular

basis for cooperativity of the catalytic site. It is also noteworthy that a single amino acid replacement can lower both the rate of multisite ATP hydrolysis and the kinetic parameters of uni-site ATP hydrolysis, suggesting that both catalyses are closely related. Finally we discussed the application of site-directed mutagenesis of amino acid residues of the β subunit identified by chemical modification.

Acknowledgments

The studies in our laboratory were supported by grants from the Ministry of Education, Science, and Culture of Japan, the Science and Technology Agency of the Japanese Government, and the Mitsubishi Foundation.

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