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MINI-REVIEW

Molecular Genetics of F_1-ATPase from *Escherichia coli*

Masamitsu Futai,[1] Takato Noumi,[1] and Masatomo Maeda[1]

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Abstract

We have reviewed recent molecular biological studies on F_1-ATPase of *Escherichia coli* and emphasized the advantages of using the bacterium in studies on this important enzyme. All subunits had homologies of varied degrees with those from other organisms. Mutations of F_1 subunits caused defects in catalysis and assembly. Defects of the mutant enzymes were studied extensively together with the determination of the amino acid substitutions. Extensive molecular biological studies may help greatly in understanding the normal mechanism and assembly of the complex.

Key Words: F_1-ATPase; H^+-ATPase; *Escherichia coli*; uni-site catalysis; *unc* operon; chemical modification; ATP synthase; ATP.

Introduction

The H^+ translocating ATPase (H^+-ATPase) in *Escherichia coli* cytoplasmic membranes catalyzes synthesis of ATP from ADP and P_i[2] at a terminal step of oxidative phosphorylation (Futai and Kanazawa, 1983; Walker *et al.*, 1984; Senior, 1985). This enzymes is a complex membrane protein similar to those found in eukaryotic organelles such as mitochondria or chloroplasts (Kagawa, 1984; Hatefi, 1985), and consists of two structurally distinct entities (or sectors) F_1 and F_0. The catalytic entity F_1 (also called F_1-ATPase) consists of five subunits α, β, γ, δ, and ε with a subunit stoichiometry of $\alpha_3\beta_3\gamma\delta\varepsilon$, and

[1] Department of Organic Chemistry and Biochemistry, The Institute of Scientific and Industrial Research, Osaka University, 8-1 Mihogaoka, Ibaraki, Osaka 567, Japan.

[2] Abbreviations: DCCD, *N,N'*-dicyclohexylcarbodiimide; DNS-ATP, 2'-(5-dimethylamino-naphthalene-1-sulfonyl)amino-2'-deoxy ATP; P_i, inorganic phosphate.

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acts as an ATPase when it is purified. The intrinsic membrane portion, F_0, functions as a proton pathway and *E. coli* F_0 has three subunits, *a*, *b*, and *c*.

Studies of *E. coli* H^+-ATPase have provided essential information on the mechanism, structure, and assembly of the entire complex and most of the results can be extended to the same enzyme in eukaryotic organelles. It must be noted that *E. coli* H^+-ATPase is the first energy-transducing ATPase whose primary structure has been determined from the DNA sequence of the cloned genes. Studies on the enzyme from this organism are advantageous, because we can easily isolate mutants, clone their alleles, and identify amino acid replacements from the codons changed by mutations. Defective properties of the mutant enzymes with defined amino acid substitutions may help greatly in understanding the normal mechanism and assembly of the enzyme. Furthermore we can apply site-directed mutagenesis to the residues replaced in mutants or those modified chemically with nucleotide analogues or general protein chemical reagents. Extensive studies along these lines may indicate the essential amino acid residues in the catalytic mechanism and subunit assembly, and these results in combination with data on the higher-ordered crystal structure will provide a final picture of this complicated enzyme.

This short article summarizes recent results on the molecular genetics of F_1-ATPase. As space is limited, we have not cited many important studies that are beyond the scope of the article. Readers are recommended to refer to articles in this issue by Fillingame on the F_0 portion and by McCarthy on gene expression of the *unc* operon coding for F_0F_1.

Unc Operon and Amino Acid Sequences of All the Subunits

After isolation of the first mutant defective in H^+-ATPase, Gibson, Cox, and co-workers established that the *unc* operon codes for this enzyme (Butlin *et al.*, 1971; Downie *et al.*, 1979). Kanazawa *et al.* (1979) found that all the structural genes are on a defined DNA segment of approximately 7 kilobase pairs corresponding to the *unc* operon and carried by a defective transducing λ phage, λ*asn*-5. The nucleotide sequence of the DNA segment was determined and information obtained from the sequence has been reviewed (Kanazawa and Futai, 1982; Futai and Kanazawa, 1983; Walker *et al.*, 1984). The order of the structural genes is *uncB*, *E*, *F*, *H*, *A*, *G*, *D*, and *C* coding for the *a*, *c*, *b*, δ, α, γ, β, and ε subunits, respectively, and the amino acid sequences of these subunits were determined from the DNA sequence (Fig. 1). Genes coding for the eukaryotic enzymes were successively cloned, mainly from organellar genomes, and sequenced as reviewed by Walker *et al.* (1984) and Futai and Kanazawa (1983). The primary sequences of bovine F_1

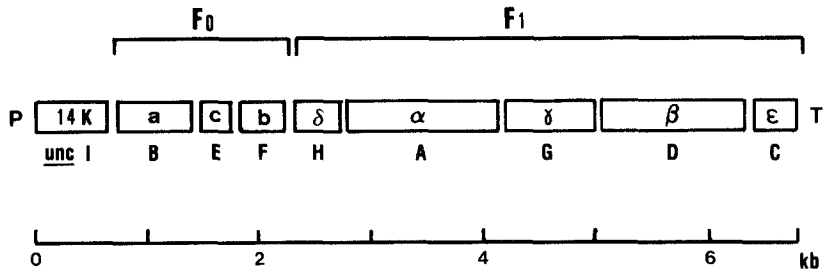

Fig. 1. Organization of the gene cluster (*unc* operon) for H^+-ATPase. Approximate positions for coding frames (cistrons) for subunits of F_1 and F_0 are shown by open boxes. A reading frame for a protein of 14,000 daltons (14 K) was found in the DNA sequence. A subunit corresponding to this reading frame was not found in the purified H^+-ATPase. An active promoter sequence (P) and a typical terminator (T) are also indicated. The scale (kb) of the gene cluster is shown in kilobase pairs.

subunits were determined by protein chemical procedures (Walker *et al.*, 1985).

The β subunit has the most conserved primary structure in different species: the primary sequence of this subunit from *E. coli* shows about 70% homology with those of chloroplasts and mitochondria, and the α subunit of *E. coli* shows about 50% homology with those of chloroplasts and bovine mitochondria. These high homologies are consistent with the conclusion that an active site is present in the β subunit or at the interface between the α and β subunits. These two subunits have regions that are homologous with those of other proteins capable of binding nucleotides, such as the mitochondrial ADP/ATP carrier, *E. coli recA* protein, and p21 ras gene product (Walker *et al.*, 1984; Futai and Kanazawa, 1983). It is especially interesting that a region of the β subunit is homologous with a sequence containing the phosphorylation site of Ca^{2+}-ATPase or Na^+/K^+ ATPase (Modyanov *et al.*, 1985). It must be noted that these two ion-transporting ATPases form an aspartyl phosphate intermediate, whereas F_1-ATPase does not form such an intermediate. The homologous region contains the aspartate (Asp-351 of Ca^{2+}-ATPase) which is phosphorylated and this residue is replaced by a threonine residue (Thr-285) in the *E. coli* β subunit. Thus it will be of interest to change Thr-285 of the β subunit to an Asp residue and study the mechanism of the altered enzyme.

The primary structures of the γ subunits of *E. coli* (Kanazawa *et al.*, 1981; Saraste *et al.*, 1981; Kanazawa and Futai, 1982), *Rhodopseudomonas blastica* (Tybulewicz *et al.*, 1984), *Rhodospirillum rubrum* (Falk *et al.*, 1985), bovine heart (Walker *et al.*, 1984), and thermophilic bacterium PS3 (Y. Kagawa, personal communication) are known and found to have considerable homology in their amino and carboxyl terminal regions. The δ subunits

show homology mainly in their carboxyl terminal regions and the ε subunits in their amino terminal regions. The δ and *b* subunits of *E. coli* have considerable homology with oligomycin sensitivity-conferring protein (OSCP) of beef heart, and the ε subunit has homology with the ATPase inhibitor of yeast or beef heart (Ernster *et al.*, 1986). These results suggest that OSCP and the δ or *b* subunit, and ε subunit and ATPase inhibitor have the same function, and possibly the same evolutional origin. Consistent with its homology with the inhibitor, the ε subunit inhibits F_1-ATPase activity (Sternweis and Smith, 1980).

Assembly of H^+-ATPase and Its Mutations

Assemblies of F_1 and F_0

All five subunits of F_1 were obtained from *E. coli* (Futai, 1977; Dunn and Futai, 1980; Sternweis and Smith, 1980). A complex formed from the α, β, and γ subunits showed ATPase activity with similar specific activity to that of the original F_1, and the entire F_1 could be reconstituted from the αβγ complex and the δ and ε subunits (Dunn and Futai, 1980). Active F_0 could be reconstituted from the isolated *a*, *b*, and *c* subunits (Schneider and Altendorf, 1985). As in *in vitro* experiments, F_1 and F_0 could be assembled independently *in vivo* as shown by Klionsky and Simoni (1985) and Aris *et al.* (1985). They introduced recombinant plasmids carrying parts of the *unc* operon into a mini-cell strain in which the *unc* operon was deleted and studied the assemblies of F_1 and F_0. Subunits of F_1 could be synthesized and assembled into an active complex in the absence of F_0 subunits, and *a*, *b*, and *c* subunits assembled into active F_0 without subunits of F_1. Similarly Fillingame *et al.* (1986) cloned the *uncB*, *E*, *F*, and *H* genes downstream of the *lac* promoter and found that cells harboring the plasmid stopped growing upon production of the H^+ pathway. Recently, Miki *et al.*[3] isolated a mutant showing temperature-sensitive translation of the γ subunit: at high temperature, membranes had no F_1-ATPase, but showed active proton translocation through F_0, whereas at low temperature, full assembly of F_0F_1 was observed. Thus active F_1 and F_0 portions can be assembled separately, at least in these experimental systems.

Cox *et al.* (1981) showed that the membranes of the polarity mutant *uncD436* did not have a functional F_0 or the α, γ, and δ subunits of F_1, but had detectable β and ε subunits. This mutation affected the two most distal genes of the operon *uncD* and *uncC* coding for the β and ε subunits,

[3]Miki, J., Maeda, M., and Futai, M., in press.

respectively. The lack of a functional F_0 was correlated with apparent absence of incorporation of the *b* subunit into the mutant membranes. After analyzing a series of assembly mutants, they suggested that the α and β subunits must be present for insertion of the *b* subunit into membranes, and presented a model of coordinated assembly of F_0F_1 *in vivo*. Their assembly model may not be valid any more, because F_1 and F_0 can be assembled independently in a plasmid-coded system, as discussed above. However, we cannot exclude the possibility that a plasmid-coded system and the chromosome-coded system have different assembly processes. As there are more than two copies of the *unc* gene in a plasmid-coded system, the amounts of subunits synthesized may be much higher than in the chromosome-coded system, where there is only one copy of the *unc* gene. An alternative interpretation of the assembly mutation may also be possible as discussed below.

We could not solubilize defective F_1 from the membranes of our assembly mutants (Kanazawa *et al.*, 1983). Wild-type F_1 could be released from membranes by washing them with dilute buffer, and the washed membranes became leaky to protons. But on the same treatment, membranes from our twelve strains defective in the β subunit released only small amounts of F_1 subunits, and the washed membranes were not permeable to protons. We are interested in these strains, because they may show defective subunit interaction(s). Senior *et al.* (1983) isolated 17 mutants defective in the β subunit and found that the membranes of more than half of them were proton-permeable, even before washing, or did not become permeable when washed to remove F_1. These mutants may be important for understanding F_0F_1 assembly and subunit interaction(s).

Analyses of Assembly Mutants

We have studied the defect(s) of our assembly mutants and determined the mutation sites (Table I) (Noumi *et al.*, 1986a). Amino acid replacements were determined from the changes of codons: Glu-41 (strain KF39), Glu-185 (KF16 and KF42), Gly-223 (KF48), and Ser-292 (KF26 and other strains) were replaced by Lys, Lys, Asp, and Phe, respectively. These mutations altered the charges, hydrophobicities, or volumes of side chains of amino acid residues in the β subunit. Glu-41, Glu-185, and Ser-292 are conserved in the β subunits of all the different species so far sequenced, and Gly-223 is conserved in the β subunit of bacteria and mitochondria, suggesting the importance of these residues. Two nonsense mutants (KF40, Gln-361 $\rightarrow$ end; KF20, Gln-397 $\rightarrow$ end) were also defective in assembly, indicating the importance of the carboxyl terminal region for subunit–subunit interaction(s). On the other hand, Gln-361 or Gln-397 itself is not an essential

Table I. Mutations in F_1 Subunits

Defect	Strain	Subunit	Codon replaced	Residue[a] replaced	Reference
I. Assembly	KF39	β	GAA(42)→AAA	Glu-41→Lys	Noumi *et al.*, 1986a
	KF16, KF42	β	GAG(186)→AAG	Glu-185→Lys	Noumi *et al.*, 1986a
	KF48	β	GGT(224)→GAT	Gly-223→Asp	Noumi *et al.*, 1986a
	KF26, KF27, KF30, KF32, KF37	β	TCC(293)→TTC	Ser-292→Phe	Noumi *et al.*, 1986a
	KF40	β	CAG(362)→TAG	Gln-361→End	Noumi *et al.*, 1986a
	KF20	β	CAG(362)→TAG	Gln-397→End	Noumi *et al.*, 1986a
	KF122	γ	ATG(1)→ATA		Miki *et al.*[c]
	KF10	γ	CAG(15)→TAG	Gln-14→End	Miki *et al.*, 1986
	KF1, KF68, KF88	γ	CAG(158)→TAG	Gln-157→End	Miki *et al.*, 1986
	KF21	γ	CAG(227)→TAG	Gln-226→End	Miki *et al.*, 1986
	KF84	γ	CAG(262)→TAG	Gln-261→End	Miki *et al.*, 1986
	KF110	γ	CAG(230)→TAG	Gln-229→End	Miki *et al.*[c]
	KF12, KF13	γ	CAG(270)→TAG	Gln-269→End	Miki *et al.*, 1986
	NR70	γ	Δ22–28	Δ21–27[b]	Kanazawa *et al.*, 1985
II. Catalysis	(uncA401)	α	TCC(373)→TTC	Ser-373→Phe	Noumi *et al.*, 1984a
	KF101	α	GCA(285)→GTA	Ala-285→Val	Soga *et al.*[d]
	KF11, KF168	β	TCT(175)→TTT	Ser-174→Phe	Noumi *et al.*, 1984b
	KF43	β	CGT(274)→CAT	Arg-246→His	Noumi *et al.*, 1986b
	KF104	β	CGT(274)→TGT	Arg-246→Cys	Kuki *et al.*[e]

[a] As indicated previously (Kanazawa *et al.*, 1981; Dunn, 1982), the isolated β and γ subunits do not have Met residues at the amino terminus, and thus residues are numbered from the Ala residue (2nd codon for both subunits).
[b] A deletion of seven amino acid residues.
[c] J. Miki, M. Maeda, M. Futai, in press.
[d] S. Soga, T. Noumi, M. Maeda, M. Futai, in preparation.
[e] M. Kuki, T. Noumi, M. Maeda, M. Futai, in preparation.

residue in the subunit, as either residue can be changed to Ser, Leu, or Tyr by introducing suppressor tRNA into the mutant. The residues or domains that are important for assembly may be limited, as independent mutants were mapped to the same residues such as Ser-292 and Glu-185.

The importance of Glu-185 or the region in its vicinity in assembly was confirmed by applying site-directed mutagenesis to this residue (Noumi *et al.*,[4] in preparation). We constructed a hybrid plasmid carrying only the *uncD* gene for the β subunit (either wild-type or mutagenized) and introduced it into a deletion strain of the *unc* operon. The properties of the β subunits could be studied using the β subunits purified from the cytoplasmic fractions, and the ATPase complex should be reconstituted from a combination of the α, β, and γ subunits, if the β is active in subunit–subunit interaction(s). Using this experimental system we showed that the β subunit with replacement of Glu-185 by Gln or Lys could not form an active complex with the α and γ subunits *in vitro*. A combination of site-directed mutagenesis and *in vitro* reconstitution may be useful for further analysis of the roles of the β subunit.

We have analyzed membrane fractions of the assembly mutants by two-dimensional gel electrophoresis, and examined the amounts of the α and β subunits and the presence of other subunits. As shown in Table II, all assembly mutants had reduced amounts of α and β subunits, suggesting that some of the subunits were dissociated from the $\alpha_3\beta_3\gamma$ type complex and partial subunit assemblies such as $\alpha_2\beta_1$ remained on the membranes. Furthermore some mutant membranes lost the ε subunit (Glu-41 → Lys) or δ and ε subunits (Glu-185 → Lys), although these membranes had the α and β

Table II. Presence and Amounts of Subunits in Membranes from Mutants Defective in the β Subunit[a]

Strain	Defect	Mutation	Presence of subunit[b]				
			α	β	γ	δ	ε
KY7230	None	None (wild)	100	100	+	+	+
KF43	Catalysis	Arg-246	94	90	+	+	+
KF39	Assembly	Glu-41	69	37	+	+	−
KF16	Assembly	Glu-185	64	36	+	−	−
KF48	Assembly	Gly-223	21	46	ND	ND	ND[c]
KF32	Assembly	Ser-292	32	23	−	+	−
KF40	Assembly	Gln-361	48	2	+	+	+
KF20	Assembly	Gln-397	43	23	+	+	+

[a] Results cited from Noumi *et al.* (1986a).
[b] Relative amounts of α and β subunits were determined immunochemically. The presence (+) or absence (−) of other subunits was determined by two-dimensional gel electrophoresis.
[c] ND, not determined.

[4] Noumi, T., Azuma, M., Maeda, M., and Futai, M., in press.

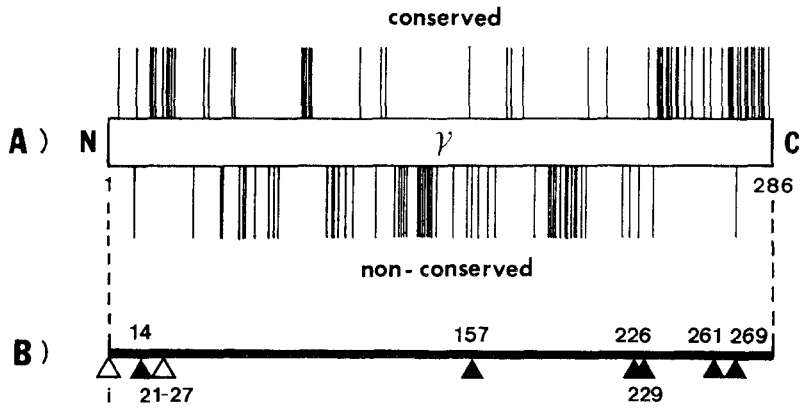

Fig. 2. Schematic illustration of the γ subunit with conserved amino acid residues and mutation sites. (A) Amino acid sequences of γ-subunits from *E. coli* (286 residues) (Kanazawa *et al.*, 1981; Saraste *et al.*, 1981; Kanazawa and Futai, 1982), *R. blastica* (286 residues) (Tybulewicz *et al.*, 1984), *R. rubrum* (299 residues) (Falk *et al.*, 1985), and bovine heart (Walker *et al.*, 1984) are aligned to obtain maximal homology. The regions deleted in the *E. coli* sequence are omitted and residues are numbered according to that of the *E. coli* sequence. The positions of identical residues in the four species are indicated by upward vertical bars on the schematic γ subunit and those of four different residues by downward vertical bars. Conserved regions are near the amino (N) and carboxyl (C) termini. (B) Positions of nonsense mutations are shown by closed triangles. The open triangles indicate the positions of a deletion (residues between 21 and 27) and of a missense mutation in the initiation codon (Met → Ile). As the Met residue is removed from the actual subunit, residues are numbered from the second codon. Cited from Miki *et al.* (1986) with some modifications.

subunits. These results cast doubt on a conceptual model of F_1 in which the $\alpha\beta\gamma$ complex is connected to F_0 through a stalk portion consisting of the δ and ε subunits (Futai and Kanazawa, 1983). The model of F_0F_1 should include direct interaction(s) between catalytic subunits (α and β) and F_0 subunits. Consistent with our observations, Aris and Simoni (1983) observed cross-linking between the β subunit and *a* or *b* subunit.

As indicated by reconstitution experiments, the γ subunit is essential in assembly of the catalytic complex: the complex with ATPase activity could be reconstituted by a combination of the α, β, and γ subunits, but not without the γ subunit (Futai, 1977; Dunn and Futai, 1980). Consistent with this observation, most mutants defective in the γ subunit were defective in assembly. We have analyzed nine mutants in the γ subunit: strain NR70 had a deletion of seven amino acid residues in the amino terminal region (Kanazawa *et al.*, 1985), and eight other strains had nonsense mutations (Miki *et al.*, 1986) (Fig. 2). One mutant strain had a replacement of an initiation codon by Val, resulting in temperature-sensitive growth on succinate (Miki *et al.*, in press). The deletion strain NR70 had only a small amount of F_1 in membranes and a significant amount of F_1 subunits in the cytoplasm.

Therefore, the seven residues in the amino terminal region may be important for assembly. As shown in Fig. 2, the nonsense mutants were capable of synthesizing a premature polypeptide of 13 (KF10), 156 (KF1, KF68, KF88), 225 (KF21), 260 (KF84), or 268 (KF12, KF13) amino acid residues. The specific ATPase activities of membranes of these strains were less than 2% of that of the wild-type. The amounts of α and β subunits found in KF12 membranes were about half those in the wild-type, and the amounts in membranes of other strains were negligible, suggesting that the region missing in KF12 [residues between 269 and 286 (carboxyl terminus)] is important for forming a stable F_1-ATPase and the regions missing in KF84 (between residues 261 and 286) is essential for assembly of the entire enzyme. Thus loss of the amino and carboxyl terminal regions resulted in defective assembly of F_1-ATPase: the missing region may actually interact with the α and β subunits. It is noteworthy that both regions are highly conserved in the γ subunits so far sequenced (Fig. 2). Similar defects in assembly seem to occur with mutations in other subunits: mutations in the α subunit also caused defective solubilization of F_1 upon washing with dilute buffer (unpublished).

Mechanism of F_1-ATPase and Its Mutations

Uni-site and Multi-site Catalyses

The F_1-ATPase has three nonexchangeable nucleotide binding sites and three exchangeable sites which form active sites (Cross and Nalin, 1982). The roles of the nonexchangeable sites in *E. coli* F_1 are unknown (Maeda *et al.*, 1976). The mechanism of hydrolysis of ATP by F_1-ATPase is "direct in-line transfer of the phosphoric residue between the ADP moiety and water," and the catalysis does not involve a phosphoenzyme intermediate (Webb *et al.*, 1980). F_1 catalyzes an exchange between water oxygen and oxygen of phosphate during hydrolysis of ATP at low concentration, but this exchange was not observed at high ATP concentration (Choate *et al.*, 1979; Hutton and Boyer, 1979). These observations suggested that ATP hydrolysis at the catalytic site is reversible and that the reversibility became lower at high ATP concentration because of the faster cooperative ATP hydrolysis. Wood *et al.* (1987) recently observed similar P_i–water oxygen exchange by *E. coli* F_1. Interestingly ε-depleted enzyme showed a much higher probability for oxygen exchange, suggesting that the ε subunit inhibits ATP hydrolysis by reducing cooperative conformational interactions that promote release of ADP and P_i.

Two types of kinetic mechanisms of ATP hydrolysis were demonstrated with F_1 of bovine heart (Grubmeyer *et al.*, 1982; Cross *et al.*, 1982)

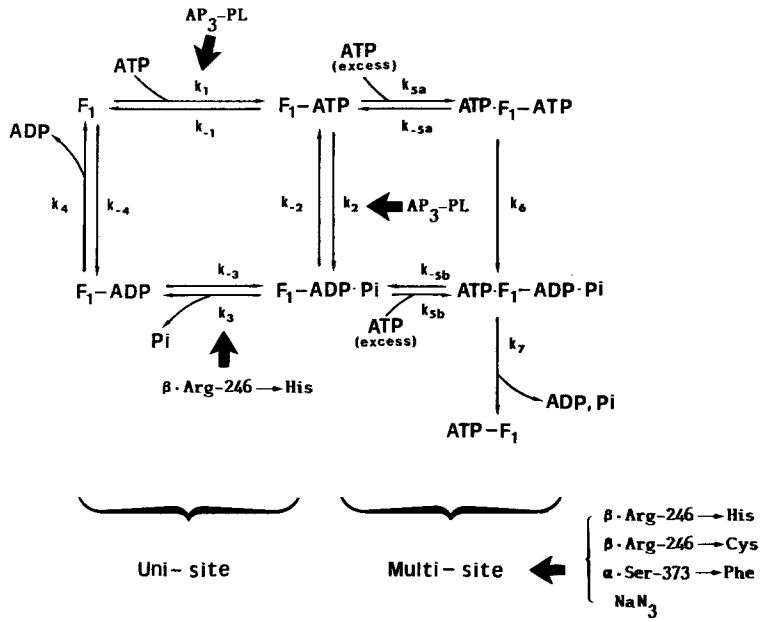

Fig. 3. Uni-site (single site) and multi-site (steady-state) catalyses of F_1-ATPase and sites of mutations and inhibitions. The scheme of ATP hydrolysis and synthesis by F_1 is cited from Penefsky and co-workers (Grubmeyer *et al.*, 1982; Cross *et al.*, 1982). Sites of mutations and actions of inhibitors are indicated. AP_3-PL, adenosine triphosphopyridoxal (Noumi *et al.*, 1987a).

and confirmed with *E. coli* F_1 (Duncan and Senior, 1985; Noumi *et al.*, 1986) (Fig. 3). When the ATP/F_1 ratio is less than 1, ATP binds at a single or first catalytic site of F_1 and is hydrolyzed only slowly ("uni-site" hydrolysis). However, in the presence of excess ATP, ATP binds to the second and third catalytic sites, ATP at the first site is hydrolyzed, and P_i is released at a rate close to the maximal velocity ("multi-site" hydrolysis). The rates of P_i release by multi-site hydrolysis (k_7) by bovine and *E. coli* F_1 are 10^6 and 10^{4-5} times, respectively, higher than that by uni-site hydrolysis (k_3). Uni- and multi-site hydrolyses can also be called single-site and steady-state hydrolysis, respectively. A slow rate of hydrolysis of an ATP analogue by *E. coli* F_1 was also observed when the analogue/F_1 ratio was close to 1 (Matsuoka *et al.*, 1982).

The equilibrium constants between $F_1 \cdot ATP$ and $F_1 \cdot ADP \cdot P_i$ of bovine and *E. coli* F_1 were 0.5–1.0, suggesting that the enzyme-bound ($F_1 \cdot ATP$) can be formed from $F_1 \cdot ADP \cdot P_i$ without energy input (Grubmeyer *et al.*, 1982; Duncan and Senior, 1985). Consistent with these kinetic results, synthesis of $F_1 \cdot ATP$ was observed with F_1-ATPase of different preparations (Feldman and Sigman, 1982; Sakamoto and Tonomura, 1983). However, these results do not indicate that energy output is not required for

the catalytic turnover (the synthesis of free ATP from free ADP and P_i). These results support the binding change mechanism of ATP synthesis (Boyer *et al.*, 1982). This mechanism includes: (i) cooperativity of the three potential catalytic sites; (ii) formation of enzyme-bound ATP without energy input, and (iii) energy-linked changes in binding affinity of ATP and ADP + P_i. At step (iii) ATP is released from the enzyme and ADP and P_i bind tightly, and, this step enables the catalytic turnover. Steps (i) and (ii) could be shown in F_1-ATPase as described above, but step (iii) is carried out by F_0F_1 coupled with an electrochemical gradient of protons. For explanation of the cooperative catalysis, other models propose that the enzyme consists of one regulatory site and two catalytic sites (Gautheron *et al.*, 1984; Pedersen and Amzel, 1985) or two regulatory sites and one catalytic site (Wang, 1985). Detailed kinetic studies using mutant enzymes may help to distinguish among these different models and to define each catalytic step.

Mutations in α and β Subunits Affecting the Catalytic Mechanism

Mutations in both the α and β subunits result in low multi-site ATPase activity (Table I). The purified mutant F_1 from *uncA401* with an amino acid replacement of αSer-373 → Phe (Noumi *et al.*, 1984a) showed low multi-site hydrolysis (less than 1% of that of the wild-type), but a normal rate in uni-site hydrolysis (Wise *et al.*, 1984; Kanazawa *et al.*, 1984). These results suggest that *uncA401* F_1 is defective in the catalytic site cooperativity that is essential for multi-site hydrolysis of ATP. The mutant F_1 showed altered ligand-dependent responses of aurovertin fluorescence (Wise *et al.*, 1984; Kanazawa *et al.*, 1984; Kanazawa and Futai, 1982). The fluorescence of aurovertin bound to the β subunit in wild-type F_1 was decreased by addition of ATP to saturate a high-affinity binding site, and this fluorescence was decreased by P_i. In contrast, the fluorescence of aurovertin bound to the β subunit of the mutant was not decreased by P_i or ATP. The fluorescence of the ATP analogue 2′-(5-dimethylaminonaphthalene-1-sulfonyl)-amino-2′-deoxy ATP bound to wild-type F_1 was increased appreciably by P_i, while the same fluorescence in the mutant F_1 was increased only slightly by P_i (Kanazawa *et al.*, 1984). These results suggest that *uncA401* F_1 is defective in transmission of conformational changes between different ligand binding sites and that Ser-373 of the α subunit or the region in its vicinity is essential for transmission of the conformational change(s) which is important in multi-site catalysis. In contrast, *uncA401* F_1 showed little P_i–water exchange during hydrolysis of 20 μM ATP hydrolysis, suggesting that the equilibrium $F_1 \cdot ATP \leftrightharpoons F_1 \cdot ADP \cdot P_i$ is shifted to $F_1 \cdot ATP$ (Wood *et al.*, 1987).

The F_1 from KF43 (βArg-246 → His) has interesting properties: multi-site ATPase activity (k_7) of the mutant enzyme was 1–3% less, but k_3 (the rate

of P_i release in uni-site hydrolysis) of the mutant was about 15-fold more than that of wild-type F_1 (Noumi *et al.*, 1987). The wild-type and the mutant F_1 had similar k_1 values (the rate of ATP binding in uni-site hydrolysis). It is interesting that the mutant enzyme shows alterations in both k_3 (P_i release in uni-site hydrolysis) and k_7 (P_i release in multi-site hydrolysis). These results suggest that a similar residue(s) or region(s) of the β subunit is related to both kinetic steps (P_i release in uni- and multi-site hydrolyses). Recently we isolated a mutant KF104 in which Arg-246 of the β subunit was replaced by Cys (Kuki *et al.*,[5] in preparation). The F_1 from this strain showed similar catalytic properties to the KF43 enzyme. F_1-ATPase from strain *uncD484* (βMet-209 → Ile) (Duncan *et al.*, 1986) also showed altered kinetics in uni-site hydrolysis and low multi-site hydrolysis, although the k_3 value of this enzyme was significantly higher than that of KF43 F_1 (Duncan and Senior, 1985). Thus Arg-246 or Met-209 or the region in its vicinity may be important in catalytic cooperativity and also in P_i binding.

As discussed above, a single amino acid replacement either in the α (Ser-373 → Phe) or β (Arg-246 → His or Cys, Met-209 → Ile) subunit resulted in loss of cooperativity and multi-site catalysis. The participation of both subunits is consistent with the suggestion that the catalytic site of F_1 is at the interface of the α and β subunits (Williams and Coleman, 1982; Bruist and Hammes, 1981). As discussed above, the hydrolysis of ATP at one catalytic site is greatly accelerated by the binding of ATP to the other catalytic sites and for this positive cooperativity of catalysis, conformational transmission between different catalytic sites is essential. As the α and β subunits are arranged alternately, conformational change of one β subunit may not be directly transmitted to other β subunits. This notion is supported by results on an *uncA* mutant defective in conformational transmission. Thus the pathway of the conformational transmission between different sites may be formed amino acid residues of both in the α and β subunits, and αSer-373, βArg-246, and βMet-209 residues may be involved in this pathway.

The residual ATPase activity of *uncA401*, its revertant (Senior *et al.*, 1984), and KF43 (Noumi *et al.*, 1986b) F_1 were resistant to azide. The wild-type enzyme was inhibited 80% by 60 μM azide (Takeda *et al.*, 1985), whereas the residual multi-site hydrolysis by KF43 was not inhibited by even 3 mM azide (Noumi *et al.*, 1987). Thus the azide binding site(s) of F_1 may be in both subunits and closely related to the catalytic cooperativity. Consistent with this possibility, azide inhibited the multi-site activity of wild-type F_1 more than 90%, but caused only slight inhibition of its uni-site hydrolysis (Noumi *et al.*, 1987b). In the presence of azide, the F_1 had a slightly lower k_1 value, but its k_{-1} and k_3 values were essentially unchanged. Azide significantly

[5] Kuki, M., Noumi, T., Maeda, M., and Futai, M., in preparation.

changed the ligand-induced fluorescence of aurovertin bound to the β subunit: Mg^{2+} slightly quenched the fluorescence of aurovertin bound to the β subunit, and this quenching was further increased by azide. These results suggest that azide inhibits catalytic cooperativity by changing site–site conformational transmission. In this regard DCCD seems to inhibit F_1-ATPase by a similar mechanism. Tommasino and Capaldi (1985) suggested that DCCD blocked cooperativity between catalytic sites: incorporation of 1 mol of DCCD per mol F_1 resulted in 95% inhibition of multi-site ATPase activity but less than 40% of the initial uni-site hydrolysis.

The F_1 from strain KF11 (*uncD11*) (βSer-174 → Phe) was different from those of the above mutants. Namely, it had substantial remaining multi-site ATPase activities, although its dependence on divalent cation was different from that of the wild-type enzyme: the ratio of Ca^{2+}- to Mg^{2+}-dependent multi-site ATPase activities in the mutant and wild-type F_1 were 3.5 and 0.8, respectively, suggesting that Ser-174 may be closely related to the Mg^{2+} binding site (Kanazawa *et al.*, 1980). The mutant F_1 was less sensitive to DCCD than the wild-type enzyme (Takeda *et al.*, 1985). DCCD binds to Glu-192 of the β subunit of the wild-type F_1 (Yoshida *et al.*, 1982) and this binding is reduced in the presence of Mg^{2+}. The presence of a Mg^{2+} binding site in the β subunit was indicated by studies the isolated subunit (Futai *et al.*, 1987): the fluorescence of 8-anilinonaphthalene-1-sulfonate bound to the isolated β subunit was increased specifically by addition of Mg^{2+}, and DCCD had no effect on the fluorescence enhancement. Tommasino and Capaldi (1985) suggested that DCCD affects the catalytic site, but mainly by blocking cooperativity between catalytic sites. The Glu-192 of the β subunit may be in the region that changes in conformation dependent on Mg^{2+}, and this region may be important for catalytic cooperativity. The mutant enzyme showed an increased P_i–water oxygen exchange reaction, suggesting that the mutation possibly stabilized the interaction of the ε subunit with the catalytic $\alpha\beta\gamma$ complex (Wood *et al.*, 1987). We have isolated the same strain (KF168, βSer-174 → Phe) from a different stock of mutants (i.e, those from which we isolated KF11), suggesting the importance of the Ser-174 residue.

Participation of Three Catalytic Sites in Multi-Site Catalysis

Consistent with the subunit stoichiometry ($\alpha_3\beta_3\gamma\delta\varepsilon$), a mechanism has been proposed in which each of the three functionally equivalent β subunits undergoes a series of conformational changes during multi-site hydrolysis of ATP (Boyer *et al.*, 1982). As discussed above, the three catalytic sites show positive cooperativity, and cleavage of ATP and release of ADP and P_i at the first catalytic site is promoted by the binding of ATP to the second and third sites. Other models proposed interaction between regulatory and catalytic

sites both in the β subunit or at the interface of the α and β subunits (Gautheron *et al.*, 1984; Pedersen and Amzel, 1985; Wang, 1985). In these cases, catalysis at the active site(s) is promoted by binding of ATP to the regulatory site(s). For either type of mechanism, it was interesting to know whether F_1-ATPase was still active in multi-site catalysis when one of its β subunits was defective in conformational transmission. To answer such question, we constructed hybrid F_1 complexes having β subunits of the wild-type and/or a mutant. As discussed above, F_1 from KF43 is defective in cooperativity between catalytic sites and thus does not show multi-site hydrolysis. We reconstituted $\alpha\beta\gamma$ complexes from the α and γ subunits of the wild-type and different ratios of β subunits from the mutant and wild-type. Analysis of the experimental results showed that the $\alpha\beta\gamma$ complex reconstituted with wild-type β subunits only was active in multi-site catalysis, whereas complexes containing one or two mutant β subunits were not active (Noumi *et al.*, 1986). Thus all three β subunits must be competent in conformational transmission for the cooperativity that is required for multi-site ATPase activity.

When one site was occupied with a substrate, it was of interest to know the kinetic properties of the other two sites. F_1 that is affinity labelled with an ATP analogue may serve as a model to study the kinetics of the remaining active sites. We tested a series of adenosine polyphosphopyridoxals and found that one of them, adenosine triphosphopyridoxal (AP_3-PL), inhibited uni- and multi-site activity almost completely upon binding of 1 mol/mol F_1: the modified F_1 had a k_1 of at least 100-fold less than that of unmodified F_1 and showed marked shift of the equilibrium $F_1 \cdot ATP \leftrightharpoons F_1 \cdot ADP \cdot P_i$ toward $F_1 \cdot ATP$ (Noumi *et al.*, 1987a). Furthermore, its uni-site hydrolysis was not stimulated by excess ATP. These results support the model in which the three catalytic sites are proposed to undergo sequential conformational changes. Similarly, Cross and Nalin (1982) reported that binding of 1 mol of adenylyl β,γ-imidodiphosphate (AMPPNP) to a single high-affinity binding site in 1 mol of mitochondrial F_1 completely inhibited multi-site ATPase activity. Binding of 5′-*p*-fluorosulfonylinosine (FSBI) to 1 mol of β subunit also completely inactivated the ATPase activity (Bullough and Allison, 1986b). On the other hand, when bound to a catalytic site, some ATP analogues are still active in cooperative conformational transmission. Chloroplast F_1 labeled with 1 mol of 2-azido ATP retains apparent catalytic cooperativity (Melese and Boyer, 1985), and all three β subunits of bovine F_1 must be labelled with 5′-*p*-fluorosulfonyladenosine (FSBA) for its complete inactivation (Bullough and Allison, 1986a). Thus the cooperative effects of these analogues bound to a catalytic site may be different from that of AP_3-PL, FSBI, or AMPPNP.

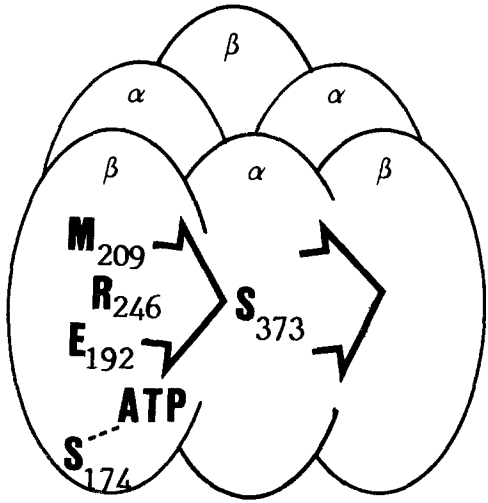

Fig. 4. Schematic model of F_1 of *E. coli*. The model of F_1 is based on the results of recent molecular biological studies. Residues shown in open arrows are those important for cooperativity.

Discussion

In this article we have reviewed recent molecular biological studies on F_1-ATPase of *E. coli*. We have emphasized the advantages of using this bacterium in studies on this important enzyme in oxidative phosphorylation. Variant enzymes with defined amino acid replacements can be obtained and studies on their defective functions and structures can contribute greatly to the understanding of the normal enzyme. Structural aspects and the catalytic mechanism of the enzyme deduced from these studies are discussed.

Figure 4 summarizes important residues for the catalytic mechanism of the enzyme. Mutations of some residues caused loss of cooperativity and we propose that these residues may form a relay system for conformational transmission. Recently Duncan *et al.* (1986) proposed a working model for the tertiary structure of the nucleotide binding domain of the β subunit of *E. coli*. Their model is consistent with the results of chemical modification studies and analysis of mutants. It is of interest that the model is consistent with residues identified in mitochondrial F_1. Two residues Lys-286 and Ile-290, identified by 8-azido-ATP labeling (Hollemans *et al.*, 1983), are close to the adenine ring of bound ATP. The proposed location of Tyr-297 (Andrews *et al.*, 1984) labeled with 4-chloro-7-nitrobenzofurazan is consistent with that deduced by inactivation studies. The residues that disrupt cooperativity when altered, including those identified by genetic studies and

DCCD binding (discussed above) are located at reasonable positions for conformational transmission between them.

It is not easy to conclude definitely whether amino acid residues that are replaced in mutant enzymes have direct roles in catalysis or assembly. Mutational replacements may change the properties of side chains of amino acid residues, leading to altered interactions with other side chains. In an extreme case, a single amino acid replacement on one side of a protein might even change the conformation of residues located on a different side of the whole molecule. Thus, at present, we can only tentatively conclude that these residues or regions in their vicinities are important for assembly or function. By analysis of the crystal structure of F_1, it will be possible to locate the catalytic residues exactly and to determine with certainty the roles of residues that are replaced by mutations, especially those that are important for cooperativity. However, even without information on the crystal structure, molecular biological approaches, including analyses of more mutants and their revertants and application of site-directed mutagenesis, seem important for further understanding of the entire assembly and the catalytic mechanism at the level of amino acid residues.

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