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Site-directed mutagenesis of stable adenosine triphosphate synthase

Masafumi Yohda [a], Shigeo Ohta [a], Toru Hisabori [b] and Yasuo Kagawa [a]

[a] *Department of Biochemistry, Jichi Medical School, Minamikawachi, Tochigi-ken, and* [b] *Department of Biology, School of Education, Waseda University, Shinjuku-ku, Tokyo (Japan)*

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Evidence was obtained that four ionizable residues in the α and β subunits of thermophilic ATP synthase (TF_0F_1), corresponding to Lys-21 and Asp-119 in the MgATP binding segments of adenylate kinase, are essential for the normal catalytic activity. TF_0F_1 was used because it is the only ATP synthase whose α-, β- and γ-subunits can be reassembled into an active complex in the absence of both ATP and Mg. Lys-164 and Asp-252 of its β-subunit were modified to isoleucine and asparagine, respectively, by site-directed mutagenesis using a multifunctional plasmid, and these genes were over-expressed in *Escherichia coli*. The resulting βI164 and βN252 subunits were both noncatalytic after re-assembly into the αβγ-complex, even though both subunits bound significant amounts of ADP. When Lys-175 and Asp-261 of the α-subunit were similarly replaced by isoleucine and asparagine, respectively, the resulting αI175 subunit reassembled weakly into an oligomer, while the αN261 subunit showed an increased dissociation constant for ADP and was reconstituted into an αβγ-complex that showed no inter-subunit cooperativity.

Introduction

ATP synthase (F_0F_1) is a major energy-supplying system of cells utilizing the protonmotive force [1]. It consists of a catalytic portion, called F_1 [2] and a proton channel portion called F_0 [3]. F_1 consists of five different subunits, α, β, γ, δ and ε, of which α and β are homologous and bind AT(D)P even after their isolation [4,5]. Based on the primary amino acid sequence of the β subunit of *Escherichia coli* F_1 (EF_1), a Rossmann fold (a supersecondary structure where a nucleotide is bound) was proposed to be the catalytic site [4]. However, F_1's obtained from thermophilic bacterium PS3 (TF_1) [5–7] and human mitochondria [8] do not contain a Rossmann fold [7,8], though the β-subunits of both EF_1 and TF_1 are interchangeable in reconstituting active hybrid αβγ-subunit complexes [9]. X-Ray crystallography and NMR spectroscopy of adenylate kinase revealed MgATP-inding segments containing Lys-21 and Asp-119 [10] which are homologous to the segments of both the α- (Lys-175 and Asp-261) and β- (Lys-164 and Asp-252) subunits of TF_1 (Fig. 1). This homology was first described in EF_1 by Walker [11]. Thus, site-directed mutagenesis of these four residues in TF_1 was attempted. TF_1 was chosen for study because it is so stable and reconstitutable [6] that non-specific denaturation by change of a residue can be avoided. The essential protonmotive activity can be measured electrically when TF_0F_1 is incorporated into a lipid bilayer [5].

Abbreviations: F_0F_1, ATP synthase; TF_0F_1, thermophilic F_0F_1; F_1, a catalytic portion of ATP synthase; TF_1, thermophilic F_1; EF_1, *Escherichia coli* F_1, 2×TY medium, 16 g bactotryptone, 10 g yeast extract and 5 g NaCl per l; UV, ultraviolet.

Correspondence: Y. Kagawa, Department of Biochemistry, Jichi Medical School, Minamikawachi-machi, Kawachi-gun, Tochigi-ken, Japan 329-04.

	21	119
Adenylate kinase	G-G-P-G-S-G-K-G-T-	-L-L-L-Y-V-D-
	175	261
TF_1 α subunit	G-D-R-Q-T-G-K-T-	-V-L-V-V-I-D-
	164	252
TF_1 β subunit	G-G-A-G-V-G-K-T-	-V-L-L-F-I-D-

Fig. 1. Lysine and aspartic acid residues in the AT(D)P binding segments of adenylate kinase and the corresponding residues in the homologous segments of the α- and β-subunits of thermophilic F_1.

Although there have been many studies on mutants of EF_1 [4,12], elucidation of the MgAT(D)P binding site has been very difficult, because both AT(D)P and Mg are strictly required for reassembly of the subunits of EF_1 into the αβγ-complex [13]. Thus, mutation of the MgATP binding site of EF_1 will result in disturbance of the reassembly, and so ATPase activity of mutant subunits cannot be tested.

TF_1 is the only F_1, reported to date, that has been reassembled from its subunits without ATP and Mg [6], and tightly bound MgATP can be removed from TF_1 without affecting its activity [5,14]. A study of the cation-dependent diastereoisomer preference of TF_1 was possible for this reason and because cadmium-ATP did not inactivate TF_1. Results showed that its true substrate is a Δ, β, γ bidentate MgATP complex [14] which is the same substrate as for crystalline adenylate kinase [10]. In contrast to other F_1's, not only αβγ, but also αβδ subunit complees TF_1 show ATPase activity [6]. As there is no homology between the γ- and δ-subunits, only the α- and β-subunits were chosen for this study.

An operon for TF_0F_1 was cloned [7,15], sequenced [7,15] and over-expressed in *E. coli* [15]. Thus, site-directed mutagenesis of the α and β subunits was possible in this study.

Materials and Methods

Materials. Oligonucleotides were synthesized with an automatic DNA synthesizer (Applied Biosystems, Model 380 B, CA, U.S.A.), according to the DNA sequence of TF_1 with desired codons at the specific sites, and purified with a high-performance liquid chromatograph (Spectra-Physics, Model 8700, CA, U.S.A.). For a mutation by base substitution, an oligonucleotide of 21 bases was used, in which the mismatch was introduced at the center of the oligonucleotide as shown in Fig. 2. Sequence primers of 17 bases were also synthesized. Nucleotides and other reagents for assay and preparation of ATP synthase were obtained as described in the previous reports [3,5,6,8]. [^{14}C]ADP was purchased from Amersham International Co., Bucks, England. Restriction endonuclease and related enzymes were obtained from Takara Shuzo, Kyoto, Japan, and Nippon Gene, Toyama, Japan [7,15–17].

Bacterial strains and plasmids. Thermophilic bacterium PS3 was originally isolated from a sample from Mine hot spring and since 1972, its ATP synthase [3,5,9,14] and its operon [7,15,16] has been analyzed. The manipulations of thermophilic DNA including sequencing were as described previously [7,15–17]. *Escherichia coli* strain NM522, genotype: recA$^+$(supE, thi, Δ(lac$^-$proAB), hsd5, {F′, proAB, lac I^q ZΔM15}) was obtained from Pharmacia, Sweden. Plasmids pTZ18R/19R [18,19] and M13 K07 helper phage which is resistant to kanamycin, were obtained from Pharmacia Japan, Tokyo. Plasmids containing DNA fragments for the α- (Hind III to Sal I, 2.3 kilobase pairs) and β- (Sac I to Sma I, 1.6 kilobase pairs) subunits were prepared as described elsehwere [15]. The manipulations of these *E. coli* strains and plasmids were as described by us [7,16,17], Maniatis et al. [18], and Davis et al. [19].

Enzyme analysis. ATPase activity was assayed at 60 °C by monitoring inorganic phosphate formation as described previously [6]. When necessary, ATPase was assayed with an ATP-regenerating system [2] to fix the ATP concentration and avoid inhibition by ADP, as described in the legend of Fig. 4. Protein concentration was measured by the method of Bradford [20]. Polyacrylamide gel electrophoresis with or without sodium dodecylsulfate was performed as reported previously [6,15,16].

Equilibrium dialysis. Equilibrium dialysis was performed at 25 °C in lucite cells (2×60 μl or 2×120 μl) using a dialysis membrane (cut-off molecular weight 7000, Biomed Instruments Inc., CA, USA) as described [21]. After incubation of

subunits with [^{14}C]ADP for 6 h to establish equilibrium, 50 μl samples were taken from each half of the cell with a microsyringe for scintillation counting [21].

Ultraviolet difference spectra. UV difference spectra were measured at 25°C with a double beam spectrophotometer Model U-3200 (Hitachi Co, Tokyo) using a pair of matched double cells with a light path of 4 mm for each compartment, as described [22,23].

Site-directed mutagenesis. The four primers (300 pmol, each) described in Fig. 2 were phosphorylated at their 5′-terminal with T4 polynucleotide kinase (four units) in a solution (50 μl) containing 1.5 μl 1 mM ATP and 5 μl of a solution of 660 mM Tris-Cl (pH 7.5), 100 mM $MgCl_2$ and 100 mM β-mercaptoethanol by incubation at 37°C for 60 min. The reaction was stopped by heating the mixture at 70°C for 10 min. Heteroduplex was formed by heating 2 μl of the 5′-phosphorylated primer (6 pmol/μl) with 2 μl of the single strand template (1 pmol/μl), 14 μl of water and 9 μl of a solution of 500 mM NaCl, 32.5 mM Tris-Cl (pH 7.5) 40 mM $MgCl_2$ and 5 mM β-mercaptoethanol at 70° for 1 h and then cooled to room temperature gradually in a heat block. This heteroduplex (13.5 μl) was polymerized and ligated with 8 units of DNA polymerase (Klenow fragment) and T4 DNA ligase (100 units) in a mixture (37 μl) of 10 μl of dNTP (2 mM each) and 2 μl of ATP (10 mM) at 12°C overnight. The yield of DNA was examined by agarose gel electrophoresis of 5 μl of the mixture after digestion of the DNA with EcoR I and Hind III. Competent *E. coli* NM522 cells were transformed with 5 μl of the solution and plated on LB plates containing 50 μg/ml ampicillin [18] at 37°C. After 10 h, about 36 colonies (diameter, 1–2 mm) were picked up with a Pasteur pipette and these transformants were infected with M13 K07 helper phage (10^7 plaque-forming units) in a final volume of 1.5 ml 2 × TY medium [18] in the presence of 70 μg per ml of kanamycin (to select cells harboring both pTZ and helper M13 phage). After 16 h incubation with shaking (160 r.p.m., culture tubes were tilted at 45°), the single strand pTZ DNA secreted into the medium was separated from the NM522 cells by centrifugation in a microfuge at 13000 r.p.m. for 5 min. The supernatant was mixed with final concentrations of 4% polyethylene glycol 6000 and 0.3 M NaCl and centrifuged in the same conditions. The pelleted phage was dissolved in 400 μl of a solution of 1% sarcosyl, 100 mM NaCl, 10 mM EDTA, 50 mM Tris-Cl (pH 8.0) and 10 μg per ml of protease K, and digested at 55°C for 1 h. Removal of contaminating protein was essential for clean DNA sequencing. The DNA in the mixture was collected by phenol treatment and ethanol precipitation as described in the manual [18]. The yield of DNA was a few microgram. The colonies were screened by DNA sequencing [7]. For identification of mutated sites, it was sufficient to sequence only one mutated base after annealing a primer ('17-mer') at about 50 base pairs downstream of the mutated site. For second screening, the mutated DNAs were prepared from positive colonies, and confirmed by sequencing four lanes of nucleotides. Competent NM522 cells were again transformed with the DNA and the resulting colonies were screened as described above.

Over-production of TF_1 subunits. Four mutated α and β subunit genes (Fig. 2) cloned in pTZ18R were expressed in *E. coli* as described for the wild-type α and β genes [15]. pTZα (mutated at I176 or N261) and pTZβ (mutated at I164 or N252) were expressed in *E. coli* NM522 in 2 × TY medium containing 100 mg per l ampicillin and 100 mg per l of isopropyl β-D thiogalactoside, in almost the same way as described for the wild type [15].

Purification of subunits. Mutated α- and β-subunits were prepared from the sonicated extracts of *E. coli* NM522 harboring pTZ18R inserted with mutated genes in almost the same way as described for wild-type subunits [15]. The elution patterns of these mutated subunits were slightly different from those of the wild-type subunits. On hydrophobic column chromatography, βN252 was eluted with 0.4 M ammonium sulfate, and βI164 with 0.55 M. For determining the affinity for nucleotide, each subunit was further purified by liquid chromatography on a gel filtration column (G3000 SW, Toyo Soda) equilibrated with 50 mM Tris-SO_4 (pH 7.5), containing 100 mM Na_2SO_4 and eluted at a flow rate of 1 ml/min.

Reassembly and purification of the αβγ-complexes. Solutions (20 μl each) of the α- (2 mg/ml),

β- (2 mg/ml) and γ- (1.64 mg/ml) subunits in 50 mM Tris-SO_4 (pH 8.0) were mixed and incubated at 25°C for at least 30 min. All the possible combinations of mutant subunits shown in Table I were obtained similarly. After incubation, 10 μl of the mixture was used for acrylamide gel electrophoresis as shown in Fig. 3, and 4 μl for ATPase assay (Table I). The values in Table I are for the mixtures, not purified complexes, and were measured in the absence of an ATP regenerating system. Kinetic analyses of these complexes were performed in the presence of an ATP regenerating system [2] as described in the legend of Fig. 4. The αβγ-complexes were purified on a gel filtration column (G3000 SW, Toyo Soda) with a liquid chromatograph (Waters model 204). The column was equilibrated with 50 mM Tris-SO_4 (pH 7.5), containing 100 mM Na_2SO_4, and the material was eluted with the same buffer at a flow rate of 1 ml/min. Absorbance was monitored with a Waters model 490 UV detector. Except for the mixture containing αI125, the active complexes were eluted at positions close to that of the wild-type αβγ-complex, and the yields of these complex were 25–55%.

Results

Mutagenesis

Replacement of Asp-261 in the α subunit by asparagine was achieved by changing the aspartate codon (GAC) of the wild-type gene to as asparagine codon (AAC) (Fig. 2). Similarly, Asp-252 of the β subunit was replaced by asparagine by changing the corresponding codon, GAT to AAT. The resulting mutants of the α- and β-subunits were named αN261 and βN252, respectively (Fig. 2). The replacements of Lys-175 of the α subunit and Lys-164 of the β-subunit by isoleucine were achieved by changing the lysine codon, AAA of each wild-type gene to the isoleucine codon (ATA). The resulting mutants of the α and β subunits were named αI175 and βI164, respectively (Fig. 2). These codon changes were introduced into the 11th nucleotides of the four synthetic '21-mers' indicated in Fig. 2. Instead of the '13-mer' usually used, we used a '21-mer' and multifunctional vector pTZ18R (2600 base pairs) to make a mutagenesis template, because in this way no subcloning was necessary and completion of primer extension was rapid. The transformants carrying the mutated plasmids were easily screened by sequencing. The use of helper phage in transformed *E. coli* NM522 harboring pTZ resulted in the secretion of single-stranded pTZ DNA, which was convenient for screening the DNA by sequencing. pTZ18R has an F1 replication origin for M13 phage DNA. Therefore, when *E. coli* NM522 harboring pTZ was infected with helper M13 K07 phage, the single-stranded pTZ DNA was packaged in M13 coat protein and was secreted from the cells. A very small amount (2% of pTZ) of the helper phage DNA was secreted from these cells. The mutation efficiencies of four primers in the first screening were only about 4%, perhaps because the extensions from the primers in most single strnaded templates were not sufficient and so most of the mutation primers were excised. However,

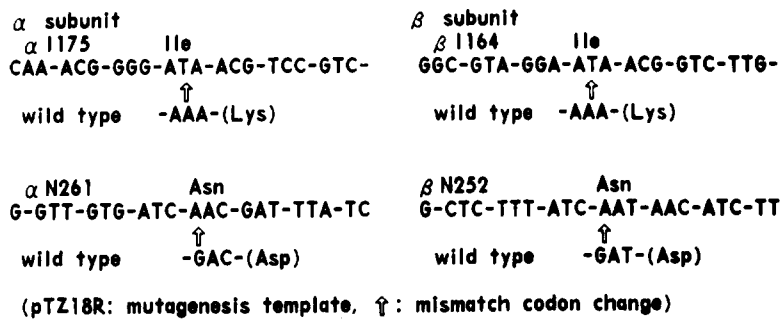

Fig. 2. Four synthetic nucleotides ('21-mer') used to induce site-directed mutagenesis of the wild type α- and β-subunits of thermophilic F_1. The four mutants thus formed are named αI175, αN261, βI164 and βN252 as indicated.

the mutation efficiencies in the second screening were more than 50%.

Reassembly of the subunits

Reassembly of the mixture of subunits took 30 min ($t_{1/2}$ was about 5 min at this high protein concentration), as reported previously [6], and unlike with EF_1 subunits, the additions of AT(D)P and magnesium were not necessary. As judged by polyacrylamide gel electrophoresis (Fig. 3) and gel filtration chromatography to purify active complexes, all the mutant subunits, except αI175, assembled to form a stable αβγ-complex or its corresponding mutated complexes, which showed identical migrations. The αI175 subunit reassembled weakly but the resulting oligomer (a faint band in Fig. 3, lane 5) was unstable and inactive.

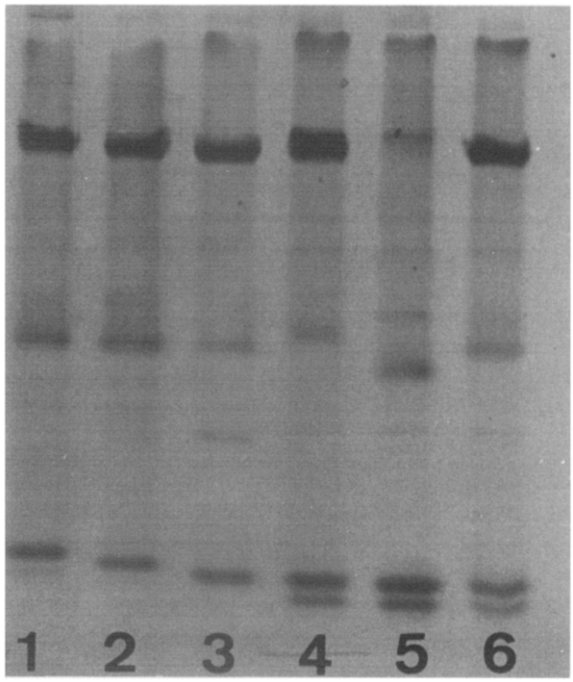

Fig. 3. Polyacrylamide gel electrophoresis of reassembled wild-type and mutant subunits. The subunits were mixed and incubated as described in the text and the mixtures (10 μg each) were subjected to gel electrophoresis without sodium dodecylsulfate as described previously [6]. After electrophoresis the gels were stained with Coomassie Blue. The arrow indicates the position of wild-type αβγ-complexes. Lane 1, wild-type α + β + γ; lane 2, α + β + γE; lane 3, α + βI164 + γ, lane 4, α + βN252 + γ; lane 5, αI175 + β + γ and lane 6, αN261 + β + γ. γE is the γ subunit of TF_1 overexpressed in *E. coli* as described in the previous paper [15].

ATPase activity of the mutated subunits

The effects of the mutations on the catalytic properties of the subunits were determined by kinetic studies on the reconstituted complexes containing the mutated subunits. Reconstitutions from all possible combinations of subunits were tested. Complex αN261-β-γ and the wild-type αβγ-complex were active, but other combinations or subunits were inactive (Table I). The apparent V_{max} of the wild-type αβγ complex and αN261-β-γ complex were nearly the same, as shown in Table I. Since the wild-type complex was strongly inhibited by the reaction product ADP, during the ATPase reaction ADP had to be removed with an ATP regenerating system [2]. Double reciprocal plots of the ATPase reaction are shown in Fig. 4. At low ATP concentrations, the K_m and K_{cat} values of αN261 were estimated to be 20 μM and 6 s^{-1}, respectively. These values were close to those of TF_1 (48 μM and 5.5 s^{-1}). However, when the ATP concentration was increased to more than 0.2 mM, the ATPase activity and K_m values of wild-type TF_1 were increased 5-fold and 20-fold, respectively (Fig. 4, lower left). In contrast to the plot for the wild-type αβγ-complex and TF_1, a

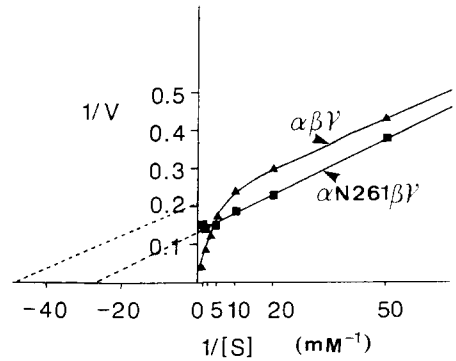

Fig. 4. Lineweaver-Burk plots of ATP hydrolysis by reconstitutd complexes. ATPase activity was measured in 1 ml of assay mixture consisting of 50 mM Tris-SO_4 (pH 8.0), 5 mM $MgSO_4$, 10 mM KCl, 5 mM phospho*enol*pyruvate, 0.32 mM NADH, 10 μg each of pyruvate kinase and lactate dehydrogenase, and the indicated concentration of ATP. The reaction was initiated by the addition of the complex at 25 °C, and the ATPase activity was calculated from the decrease of absorbance at 340 nm in steady-state conditions. [S], ATP concentration in mM; *V*, velocity of ATP hydrolysis in mol ATP hydrolyzed per mol TF_1 or equivalent αβγ complex per s. The molar weight of TF_1 was 385 351.

TABLE I

ATPASE ACTIVITIES OF REASSEMBLED MIXTURES OF WILD-TYPE AND MUTANT SUBUNITS

The subunits were mixed, incubated in the absence of MgATP [6] and assayed as described in the text. Purified subunits and other combinations not indicated in this table were all inactive.

α-Subunit			β-Subunit			γ-Subunit	ATPase
α	αI175	αN261	β	βI164	βN252	γ	(μmol/mg)
+			+			+	1.81
+							0.01
			+			+	0.02
			+				0.01
		+	+			+	1.41
		+					0.00
+				+		+	0.00
				+			0.02
+					+	+	0.01
					+		0.02
			+			+	0.02
+						+	0.02
	+		+			+	0.02

double reciprocal plot for the αN261-β-γ complex was linear (Fig. 4). The initial lag phase of the ATPase reaction seen with the wild-type TF_1, was not detected with the αAsn261-β-γ complex. These results suggest that in this mutant, cooperativity between nucleotide binding sites was lost.

ADP-binding activity of the mutated subunits

Since ADP is the substrate for ATP synthase as well as being a strong regulator of ATP hydrolysis, the ADP-binding activity of each subunit was measured by UV-difference spectroscopy (Fig. 5) and equilibrium dialysis with [^{14}C]ADP (Table II). The affinities of the mutant β-subunits were partly conserved with or without Mg, and the UV difference spectra showed identical absorption maxima to that of the wild-type β (Fig. 5). Owing to the reduced ADP-binding activity of the mutant α-subunits, the K_d values were not accurate, especially in the absence of Mg. Although K_d values

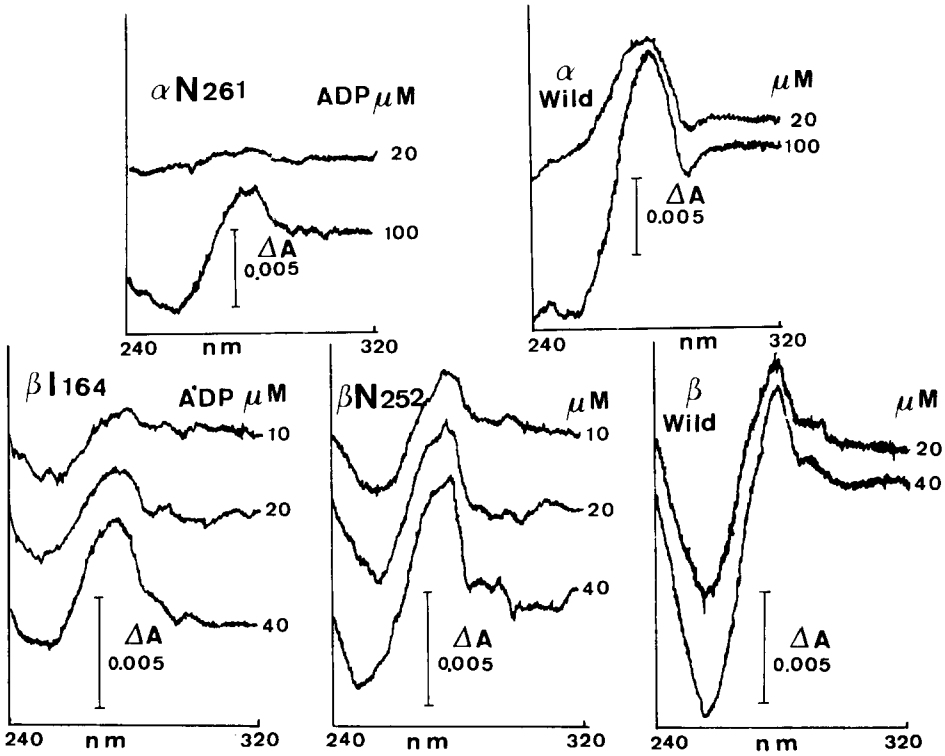

Fig. 5. UV difference spectra for the interaction of ADP with the isolated wild type and mutated subunits of TF_1. The indicated concentrations of ADP and 2 mM $MgCl_2$ were added to 8.0 μM of the αN261 and wild-type α-subunits (upper), and 7.5 μM of the βI164, βN252 and wild-type β-subunits (lower) in 50 mM Tris-SO_4, 100 mM Na_2SO_4 (pH 7.5) at 25 °C, and spectral change was recorded 10 min later, as described previously [22]. The base line of each difference spectrum was the value around the wave length of 320 nm and each curve was obtained by computer averaging of the absorbance data.

TABLE II

K_d's OF MUTATED TF_1 SUBUNITS FOR ADP DETERMINED BY EQUILIBRIUM DIALYSIS WITH [^{14}C]ADP

The binding of radioactive ADP to the subunits was measured as described previously [21] in the conditions described in the text.

α-Subunit	Mg	K_d (μM)	β-Subunit	Mg	K_d (μM)
α wild-type	+	5.2 [a]	β wild-type	+	25 [a]
α wild-type	−	> 100 [a,b]	β wild-type	−	24 [a]
αI175	+	100–200 [b]	βI164	+	55–80
αI175	−	no binding	βI164	−	65–80
αN261	+	65–85	βN252	+	30–50
αN261	−	no binding	βN252	−	45–60

[a] Values from reference [23].
[b] [^{14}C]ADP was bound but no saturation was observed.

estimated by the difference absorption spectra (Fig. 6) are rather lower than those obtained by equilibrium dialysis (Table II), the ADP-binding activities of the wild-type β and βN252 were higher than that of βI164, and the activity of wild type α was higher than those of αN261 and αI175 in the presence of Mg.

Discussion

This work describes the site-directed mutagenesis of the nucleotide binding subunits (i.e., the α- and β-subunits) of thermophilic ATP synthase (TF_0F_1). Use of this system enabled us to distinguish the catalytic activity (lost in the βI164 and βN252 subunits) (Table I) from ability to reassemble without MgAT(D)P (Fig. 3). The catalytic activity was also separated from ADP-binding activity remaining in the same mutant subunits (Fig. 5). This fact also excluded the possibility that the loss of catalytic activity was caused by nonspecific denaturation of the mutant subunits. The loss of cooperativity in the catalytic activity of the αN261-β-γ subunit complex (Fig. 4), and decreased ability to reassemble in αI175 (Fig. 3), suggested a role of the mutated residues of the α-subunit in inter-subunit interaction.

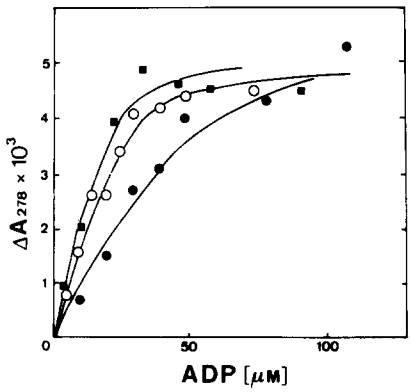

Fig. 6. Dependence of UV spectral change of isolated subunits on ADP concentration. Isolated subunits (7.5 μM of the wild-type and mutant β-subunits) were incubated with the indicated amounts of ADP in 50 mM Tris-SO_4, 100 mM Na_2SO_4 (pH 7.5) and 2 mM $MgSO_4$ at 25 °C, as described previously [22]. Open circles, βN252; closed circles, βI164, and squares, wild-type β-subunit.

Reassembly of the α-, β- and γ-subunits without ATP

Since both AT(D)P and Mg are essential for reconstitution of the *E. coli* αβγ-complex [13], it is very difficult to distinguish a mutant deficient in MgAT(D)P-binding from one deficient in assembly. The TF_1 subunits of the wild type and mutants did not, however, require MgAT(D)P for reasembly, and as shown in Fig. 3, reassembly of all the mutant subunits, except αI175, was observed. The αI175-β-γ complex was unstable, perhaps because of reduced inter-subunit interaction related to the mutated nucleotide binding site.

Nucleotide binding to subunits

We expected that replacement of residues in the AT(D)P binding site would result in loss of ADP binding. However, as shown in Figs. 5 and 6 and in Table II, mutant subunits showed considerable ADP-binding activity. Fig. 5 shows a red-shift of the absorption maximum of the adenine por-

tion which is surrounded by a hydrophobic environment as reported previously [21–23]. Therefore, there must be segments which bind ADP hydrophobically in those subunits. In fact, X-ray crystallography of the nucleotide-adenylate kinase complex revealed not only the segments shown in Fig. 1, but also a segment (Ile-28 and Val-29) constituting a hydrophobic pocket [10]. The homologous residues corresponding to these segments in TF_1 are Ala-180 and Leu-181 of the α subunit and Leu-171 and Ile-172 of the β-subunit, which remained intact after the mutagenesis.

Since AMP was not bound to these subunits [21–25], polyphosphate binding residues may also be important. The replacement of ionizable residues in the AT(D)P binding site by nonionizable residues reduced the affinity (Table II). X-Ray crystallography of tyrosyl tRNA synthase [26] and a GTP-binding protein [27] have revealed the residues that interact directly with polyphosphate. Adenylate kinase (Fig. 1), GTP-binding proteins and several other enzymes contain segments that are homologous to the α- and β-subunits of F_1's from many sources [4,7,10,28], whereas in tyrosyl tRNA synthase the residues interacting with phosphate are very different (His-45, His-48, Thr-40 and Thr-51) [26]. Thus, AT(D)P binding site itself may not have a strict residue requirement.

One role of the lysine containing segment I of adenylate kinase (Fig. 1) is assumed to be relocation of catalytic groups towards the reaction center of the bound substrate [10]. This possibility would explain the loss of ATPase activity (Table I) without complete loss of ADP binding activity by βI164 and βN252 (Fig. 6).

Effect of Mg on ADP binding

Asp-119 of adenylate kinase (Fig. 1) is closely related to magnesium binding [10]. However, the replacement of Asp-252 by Asn-252 did not change the effect of Mg on ADP binding (Table II). Despite the presence of homologous aspartate residues in the ATP-Mg binding sites of the α- and β-subunits (Fig. 1), Mg was required only for AT(D)P binding to the α-subunit [5,21] (Table II). Although Mg was not required for AT(D)P binding to the β-subunit, the true substrate of TF_1 was shown to be a Δ, β, γ bidentate MgATP complex [14], and the synthesis of the ATP-TF_1 complex from the ADP-TF_1 complex required P_i and Mg [29]. The loss of ATPase activity of βN252 might be explained by the hypothesis that the homologous Asp-119 of adenylate kinase facilitates the migration of Mg from β,γ-coordination in MgATP to α,β-coordination in MgADP [10].

Role of nucleotide bound to the α-subunit

The presence of ATPase activity of the αN261-β-γ complex (Fig. 4) suggests that the α-subunit has no catalytic role. A regulatory role of the α-subunit was shown with a mutant defective in the α subunit of EF_1 [30]. This mutant EF_1, like the αN261-β-γ subunit, slowly hydrolyzes ATP at the first catalytic site (uni-site hydrolysis proposed by Penefsky [31]), but the enhancement of its ATPase activity at higher ATP concentration (multi-site catalysis [31]) was only 1% of that of the wild-type F_1 [30]. Since the multisite catalysis is due to inter-subunit interaction, a mutant of the β-subunit (Arg-246 replaced by His) of EF_1 also showed loss of this multi-site activity [32]. The strong AT(D)P binding of the α subunit of EF_1 [4,13] was explained by the presence of an arginine residue in the α subunit instead of alanine in the β-subunit (Fig. 1, third residues from the left) [10]. However, the same residues are present in TF_1, and yet both MgATP [33] and MgADP [23,33] bind to both the α- and β-subunits of TF_1 [34,23]. The ATPase reaction of F_1 is controlled allosterically by the ATP concentration (Fig. 4, wild-type TF_1), whereas that of ITPase follows a linear Linewaver-Burk curve [35]. In accordance with this observation, IT(D)P binds strongly to the β-subunit but very weakly tot he α-subunit [34]. ITPase of F_1 is strongly inhibited by ADP [2]. In this work, we found that αN261 showed decreased affinity to ADP. Results by infrared spectroscopy (hydrogen exchange method) indicated that nucleotide affected the conformation of the β-subunit only in the presence of α subunit [24]. The results on αN261 are consistent with the idea of a regulatory role of the α-subunit.

After this manuscript was completed, an *E. coli* mutant (not by site-directed mutagenesis) was reported which has a point mutation (Ala-151 to Val) at the conserved sequence GXXXXGKT/S (Fig. 1) in the β-subunit [36]. The authors group also reported two mutants (Glu-190 to Gln and

Glu-201 to Gln) of the TF_1 β-subunit which were also inactive after reconstitution with the α- and γ-subunits [37].

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References

1 Mitchell, P. (1985) J. Biochem. 97, 1–18.
2 Pullman, M.E., Penefsky, H.S., Datta, A. and Racker, E. (1960) J. Biol. Chem. 235, 3322–3329.
3 Okamoto, H., Sone, N., Hirata, H., Yoshida, M. and Kagawa, Y. (1977) J. Biol. Chem. 252, 6125–6131.
4 Futai, M. and Kanazawa, H. (1983) Microbiol. Rev. 47, 258–312.
5 Kagawa, Y., Hirata, H., Ohta, S., Ishizuka, M. and Karube, I. (1987) Ion Transport Through Membranes (Yagi, K. and Pullman, B., eds.), pp. 147–162, Academic Press, New York.
6 Yoshida, M., Sone, N., Hirata, H. and Kagawa, Y. (1977) J. Biol. Chem. 252, 3480–3485.
7 Kagawa, Y., Ishizuka, M., Saishu, T. and Nakao, J. (1986) J. Biochem. 100, 923–934.
8 Ohta, S. and Kagawa, Y. (1986) J. Biochem. 99, 135–141.
9 Takeda, K., Hirano, M., Kanazawa, H., Nukiwa, N., Kagawa, Y. and Futai, M. (1982) J. Biochem. 91, 695–701.
10 Fry, D.C., Kuby, S.A. and Mildvan, A.S. (1986) Proc. Natl. Acad. Sci. USA 83, 907–911.
11 Walker, J.E. and Gay, N.J. (1984) Biochim. Biophys. Acta 768, 164–200.
12 Noumi, T., Oka, N., Kanazawa, H. and Futai, M. (1986) J. Biol. Chem. 261, 7070–7075.
13 Dunn, S.D. and Futai, M. (1980) J. Biol. Chem. 255, 113–118.
14 Senter, P., Eckstein, F., and Kagawa, Y. (1983) Biochemistry 22, 5514–5518.
15 Ohta, S., Ishizuka, M., Yohda, M., Hirata, H., Hamamoto, T., Otawara-Hamamoto, Y., Matsuda, K. and Kagawa, Y. (1988) Biochim. Biophys. Acta 933, 141–155.
16 Saishu, T., Nojima, H. and Kagawa, Y. (1986) Biochim. Biophys. Acta 867, 97–106.
17 Kagawa, Y., Nojima, H., Nukiwa, N., Ishizuka, M., Nakajima, T., Yasuhara, T., Tanaka, T. and Oshima, T. (1984) J. Biol. Chem. 59, 2956–2960.
18 Maniatis, T., Fritsch, E.F. and Sambrook, J. (1982) Molecular Cloning: a Laboratory Manual, Cold Spring Harbor, New York.
19 Davis, L.G., Dibner, M.D., and Battey, J.F. (1986) Basic Methods in Molecular Biology, Elsevier, Amsterdam.
20 Bradford, M. (1976) Anal. Biochem. 72, 248–258.
21 Hisabori, T. and Sakurai, H. (1984) Plant Cell Physiol. 25, 483–493.
22 Tanaka, M. and Sakurai, H. (1980) Plant Cell Physiol. 21, 1585–1593.
23 Hisabori, T., Yoshida, M., and Sakurai, H. (1986) J. Biochem. 100, 663–670.
24 Ohta, S., Tsuboi, M., Yoshida, M. and Kagawa, Y. (1980) Biochemistry 19, 2160–2165.
25 Kagawa, Y. and Yoshida, M. (1979) Methods Enzymol. 55, 781–787.
26 Fersht, A.R., Leatherbarrow, R.J. and Wells, T.N.C. (1986) Trends Biochem. Sci. 11, 321–325.
27 Bourne, H.R. (1986) Nature 321, 814–817.
28 Walker, J.E., Fearnley, I.M., Gay, N.J., Gibson, B.W., Northrop, F.D., Powell, S.J., Runswick, M.J., Saraste, M. and Tybulewicz, V.L.J. (1985) J. Mol. Biol. 184, 677–704.
29 Yohda, M., Kagawa, Y. and Yoshida, M. (1986) Biochim. Biophys. Acta 850, 429–438.
30 Wise, J.G., Latchney, L.R., Ferguson, A.M. and Senior, A.E. (1984) Biochemistry 23, 1426–1432.
31 Cross, R.L., Grubmeyer, C. and Penefsky, H.S. (1982) J. Biol. Chem. 257, 12101–12105.
32 Noumi, T., Taniai, M., Kanazawa, H. and Futai, M. (1986) J. Biol. Chem. 261, 9196–9201.
33 Roegner, M., Graeber, P., Luecken, U., Tiedge, H., Weber, J. and Schaefer, G. (1986) Biochim. Biophys. Acta 849, 121–130.
34 Ohta, S., Tsuboi, M., Ohshima, T., Yoshida, M. and Kagawa, Y. (1980) J. Biochem. 87, 1609–1617.
35 Ebel, R.E., and Lardy, H.A. (1975) J. Biol. Chem. 250, 191–196.
36 Hsu, S.-Y., Noumi, T., Takeyama, M., Maeda, M., Ishibashi, S. and Futai, M. (1987) FEBS Lett. 218, 222–226.
37 Ohtsubo, M., Yoshida, M., Ohta, S., Kagawa, Y., Yohda, M. and Date, T. (1987) Biochem. Biophys. Res. Commun. 146, 705–710.