

β -Subunit of *Escherichia coli* F₁-ATPase

An amino acid replacement within a conserved sequence (G-X-X-X-X-G-K-T/S) of nucleotide-binding proteins

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A mutant strain KF87 of *E. coli* with a defective β -subunit (Ala-151 \rightarrow Val) of F₁-ATPase was isolated. The mutation is within the conserved sequence (G-X-X-X-X-G-K-T/S) of nucleotide-binding proteins. The mutant F₁-ATPase had a much higher rate of uni-site hydrolysis of ATP than the wild type, and about 6% of the wild-type multi-site activity. The mutant enzyme showed defective transmission of conformational change(s) between the ligand- and aurovertin-binding sites.

F₁-ATPase; Nucleotide-binding protein; Uni-site catalysis; Oncogene

1. INTRODUCTION

The H⁺-ATPase of *E. coli* catalyzes synthesis of ATP coupled to an electrochemical gradient of protons like the H⁺-ATPases in mitochondria and chloroplasts (reviews [1–5]). The catalytic portion F₁ (or F₁-ATPase) is formed from five subunits, and the catalytic site is believed to be in the β -subunit or at the interface between the α - and β -subunits.

The sequences of all the subunits of *E. coli* H⁺-ATPase have been established by DNA sequence analysis of the *unc* operon coding for this enzyme. The subunit sequences of other bacterial and eukaryotic enzymes have been deduced from cloned DNA [1,2] or obtained directly by protein sequencing [6]. The β -subunit is highly conserved

in different organisms and the sequence G-X-X-X-X-G-K-T/S-X-X-X-X-X-X-I/V is found in the β -subunits, adenylate kinase, and other nucleotide-binding proteins [7–9] (table 1). Adenylate kinase is the only protein in which the ATP-binding site has been studied in detail [8,10]: residues 15–22 (G-G-P-G-S-G-K-G) of this enzyme form a flexible loop structure between an α -helix and β -sheet, and Lys-21 (K) may be very close to the α - [8] or γ -phosphate [10] of ATP. ATPase activity of the mitochondrial F₁ is lost by a modification of the corresponding Lys residue with 7-chloro-4-nitrobenzofrazan [11]. Residues 149–156 (G-G-A-G-V-G-K-T) of the *E. coli* β -subunit may have a similar role.

The roles of specific amino acid residues in F₁-ATPase can be analyzed by studying the properties of the mutant enzymes, and such studies have suggested the importance of Ser-373 [12] of the α -subunit and Ser-174 [13] and Arg-246 [14] of the β -subunit in the catalysis. Here, we identified a mutant in which the β -subunit had a substitution

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Table 1
Conserved sequence of nucleotide-binding proteins

Nucleotide-binding protein	Sequence	References
Conserved sequence	<u>G X X X X G K T / S</u>	
<i>E. coli</i> F ₁ - β (wild type)	G G <u>A</u> G V G K T	(149-156) 21
(KF87)	G G <u>V</u> G V G K T	(149-156) this paper
<i>E. coli</i> F ₁ - α	G D R G T G K T	(169-176) 29
Normal p21 <i>ras</i>	G A G G V G K S	(10-17) 25,26
Activated p21 <i>ras</i>	G A V G V G K S	(10-17) 25,26
Adenylate kinase (porcine)	G G P G S G K G T	(15-23) 30
EF-Tu (<i>E. coli</i>)	G H V D H G K T	(18-25) 31

The conserved sequence G-X-X-X-G-KT/S-X-X-X-X-X-I/V has been found in more than 40 nucleotide-binding proteins [7,8,32]. Examples are shown here together with the mutation of the β -subunit found in the present study

(Ala-151 \rightarrow Val) within the conserved region discussed above. Consistent with the proposed role of this sequence, this mutant F₁ showed altered catalytic properties.

2. EXPERIMENTAL

E. coli strains KY7230 (*asn*, *thi*, *thy*) and KF43 (*uncD43*, *thi*, *thy*) [14] were used. Strain KF87 (*uncD87*) with a defective β -subunit was isolated as described [15]. Minimal medium supplemented with thymine, thiamine, asparagine and a carbon source (glucose, glycerol or succinate) and a rich medium (L-broth) with 20 μ g/ml of ampicillin or tetracycline were used for genetic analysis (agar plates) and preparation of membranes [14]. For complementation or recombination tests, competent cells were mixed with plasmid DNA and spread over L-broth agar plates containing antibiotics [14]. By use of hybrid plasmids pFT1502, pTN1666 and pFT1503 [14], the *uncD87* mutation was mapped on a 790 bp DNA segment with the *EcoRI* and *PstI* sites at the promoter proximal and distal ends, respectively.

Chromosomal DNA of mutant strain KF87 was prepared, digested with *EcoRI* and *PstI* endonucleases and subjected to electrophoresis on polyacrylamide gel [14]. The fraction of DNA fragments corresponding to 790 bp was eluted from the gel matrix and ligated with pBR322 which had been digested with the corresponding endonucleases. The recombinant plasmids were then introduced into strain KF43 (Arg-246 \rightarrow His)

[14]. Cells with the plasmid (pKF87) carrying the mutant gene could grow on succinate after genetic recombination. Other procedures were as described in [13,14].

Multi-site (steady-state) ATPase activity was assayed at 25°C by incubating 0.20 μ M F₁ with 4.0 mM [γ -³²P]ATP (3×10^{-4} Ci/mmol) and 2.0 mM MgCl₂ in 40 mM Tris-HCl, pH 8.0 [16]. Routinely, ATPase was also assayed at 37°C and 1 unit of the enzyme was defined as the amount hydrolyzing 1 μ mol ATP/min [17]. Uni-site (non-steady-state) hydrolysis of ATP was assayed essentially as in [18] by incubating [γ -³²P]ATP (0.25 μ M) with 0.5 μ M F₁ at 25°C. Release of ³²P_i, bound [γ -³²P]ATP and ³²P_i, or release of [γ -³²P]ATP was assayed according to [14,18].

Membranes [19] and wild-type F₁ [17] were prepared from cells that had been passed through a French press. The mutant F₁ could be isolated from 20 g of wet cells with similar recovery and purity by a slight modification of the procedure used for the wild-type F₁. Formation of an electrochemical gradient of protons [19] and amounts of protein [20] were assayed using published procedures. Aurovertin was a gift from Dr M.G. Douglas. Other reagents used were as described in [14] or of the highest grade available commercially.

3. RESULTS AND DISCUSSION

The DNA fragment carrying the mutant allele of strain KF87 was cloned. On sequencing the two

strands of the 790 bp DNA fragment, a change from GCG to GTG was found in the 152nd codon. This mutation results in the replacement of Ala-151 by Val in the β -subunit (the isolated β -subunit has no Met at its amino-terminus [21]). The Ala residue is conserved in the β -subunits of different origins, and within the conserved sequence of nucleotide (ATP) binding proteins (table 1), G-G-A-G-V-G-K-T (conserved residues are underlined; residues 149–156 of the *E. coli* β -subunit).

Membranes of the mutant retained 6–8% of the ATPase activity of the wild type and could not form a pH gradient dependent on ATP. The specific activity of the mutant F_1 was about 6 U/mg protein and its K_m was 0.75 mM under steady-state assay conditions keeping the Mg^{2+} /ATP ratio constant (1:2), whereas wild-type F_1 had a specific activity of about 90 U/mg protein and a K_m of 0.20 mM [17]. The residual activity of the mutant F_1 had the same sensitivity as the wild type to azide, quercetin and 4-chloro-7-nitro-1,2,3-benzoxadiazole, but it was less sensitive than the latter to dicyclohexylcarbodiimide (DCCD): 50 μ M DCCD caused 70% inhibition of the mutant enzyme, but complete inhibition of the wild-type enzyme. As shown previously [16], azide does not inhibit the initial rate of uni-site hydrolysis, suggesting that the remaining activity of the mutant F_1 is not due to uni-site hydrolysis.

The mutant and wild-type F_1 s had similar high-affinity K_d values for aurovertin (about 1.1 and 0.7 μ M, respectively, assayed by fluorescence increase), suggesting that they had similar aurovertin-binding sites in their β -subunits [22]. The fluorescence of aurovertin bound to the wild-type F_1 was enhanced by ADP (fig.1A); it first increased rapidly and then decreased gradually after addition of ATP and Mg^{2+} (fig.1C), whereas the fluorescence of aurovertin bound to the mutant F_1 was not affected by these ligands (fig.1B,D). These results suggest that the mutant F_1 has a defect in transmission of conformational changes between the ligand-binding site and the aurovertin site in the β -subunit.

As shown first for the bovine F_1 [23,24] and later for the *E. coli* F_1 [14,18], $[\gamma\text{-}^{32}\text{P}]\text{ATP}$ is hydrolyzed only slowly by 'uni-site hydrolysis', but addition of excess unlabelled ATP promotes hydrolysis of $[\gamma\text{-}^{32}\text{P}]\text{ATP}$ and the release of the products $^{32}\text{P}_i$

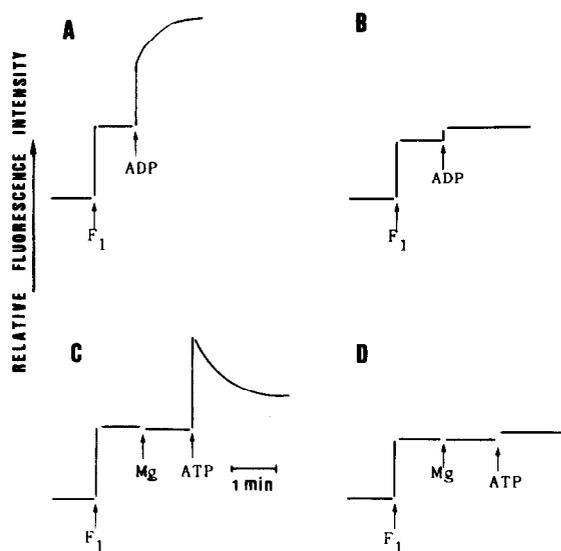


Fig.1. Absence of ligand-dependent fluorescence change of aurovertin in mutant F_1 . F_1 (0.38 μ M) of the wild type (A,C) and mutant (B,D) was added to 10 mM Tris-HCl, pH 8.0 (1.0 ml), containing 1.0 μ M aurovertin, 0.25 M sucrose and 0.5 mM EDTA and the fluorescence change (excitation, 365 nm; emission, 470 nm) was monitored [16]. The following compounds were added at the times indicated by arrows: 0.1 mM $MgCl_2$ (Mg), 100 μ M ADP and 100 μ M ATP (final concentrations). Corrections were made for slight shifts of the baselines upon addition of these compounds in each experiment.

and ADP from the first catalytic site (cold chase). The initial rate of uni-site hydrolysis by the mutant enzyme (determined by measuring the $^{32}\text{P}_i$ bound to F_1 and also that released into the medium) was higher than that of the wild type: the values for the mutant and wild-type F_1 s were ≥ 0.08 and 0.01 mol/mol F_1 per s, respectively (fig.2). With both enzymes, the rate was insensitive to sodium azide, confirming previous findings [16]. The values for k_1 (rate of $[\gamma\text{-}^{32}\text{P}]\text{ATP}$ binding) of the wild-type and mutant F_1 s were 6.0×10^4 and $\geq 33 \times 10^4 \text{ M}^{-1} \cdot \text{s}^{-1}$, respectively. Values for k_{-1} (rate of release of $[\gamma\text{-}^{32}\text{P}]\text{ATP}$) and k_3 (rate of release of $[\gamma\text{-}^{32}\text{P}]\text{P}_i$) could not be measured because the amounts of $[\gamma\text{-}^{32}\text{P}]\text{ATP}$ and $[\gamma\text{-}^{32}\text{P}]\text{P}_i$ bound to the mutant F_1 were too low (fig.3): the k_3 value of the mutant enzyme was at least 50–100-fold higher than that of the wild type. It should be noted that cold chase had no effect on the uni-site hydrolysis by the mutant enzyme

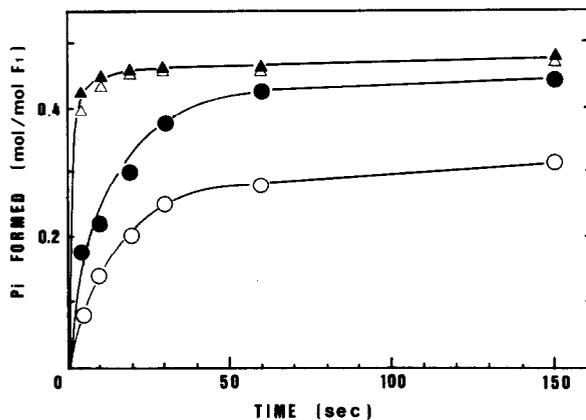


Fig. 2. Uni-site hydrolysis of ATP by F_1 -ATPase from the mutant KF87 and wild type. Mixtures of $0.25 \mu\text{M}$ $[\gamma\text{-}^{32}\text{P}]\text{ATP}$ and $0.5 \mu\text{M}$ F_1 of the mutant (Δ , \blacktriangle) or wild type (\circ , \bullet) in 50 mM Tris- SO_4 , pH 8.5, containing 1 mM K_2HPO_4 and 0.5 mM MgSO_4 were incubated for the indicated times. Reactions were terminated by addition of perchloric acid (\circ , Δ) (acid quench) or 1 min after dilution with a 2×10^4 -fold molar excess of non-radioactive ATP (\bullet , \blacktriangle) (cold chase).

(fig. 2, \blacktriangle); the equilibrium $F_1 \cdot [\gamma\text{-}^{32}\text{P}]\text{ATP} \rightarrow F_1 \cdot \text{ADP} \cdot [\gamma\text{-}^{32}\text{P}]\text{P}_i$ was shifted toward $F_1 \cdot \text{ADP} \cdot [\gamma\text{-}^{32}\text{P}]\text{P}_i$. These results indicate that the mutant enzyme binds $[\gamma\text{-}^{32}\text{P}]\text{ATP}$ and releases $[\gamma\text{-}^{32}\text{P}]\text{P}_i$ much faster than the wild type and shows a greatly increased rate of uni-site hydrolysis. The promotion of catalysis of the mutant enzyme calculated from the ratio of the rates of release of $[\gamma\text{-}^{32}\text{P}]\text{P}_i$ in multi-site (k_7) and uni-site (k_3) hydrolyses is much lower than that of the wild type.

Here, we have shown that replacement of Ala-151 by Val in the β -subunit results in catalytic defects in F_1 : the mutant enzyme has a higher uni-site activity, although its multi-site activity is 6–8% of that of the wild-type enzyme.

Ala-151 is within the sequence G-X-X-X-X-G-K [7,8] found in the ATP-binding region of adenylate kinase and conserved in nucleotide-binding proteins. It is interesting that normal and activated (oncogenic) p21 *ras* proteins have Gly and Val residues (table 1), respectively [25,26], at the position corresponding to Ala-151 of the β -subunit. Like the mutant F_1 -ATPase, the oncogenic protein has lower GTPase activity than normal (about 10% of normal), but normal GTP-binding activity [27]. These results support the idea

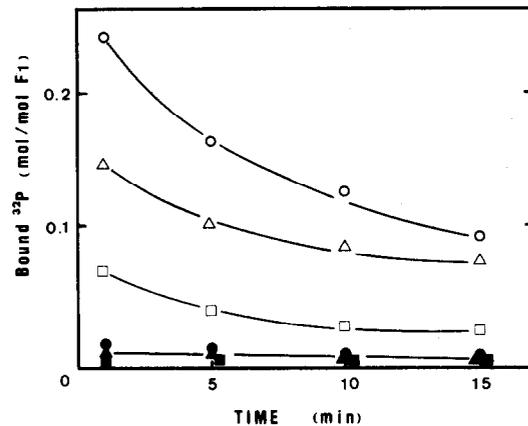


Fig. 3. Bound $[\text{}^{32}\text{P}]\text{ATP}$ and $^{32}\text{P}_i$ during uni-site hydrolysis by the mutant and wild-type F_1 s. $0.25 \mu\text{M}$ $[\gamma\text{-}^{32}\text{P}]\text{ATP}$ was incubated with $0.5 \mu\text{M}$ of the mutant (\bullet , \blacktriangle , \blacksquare) or wild-type (\circ , Δ , \square) F_1 . At the indicated times, aliquots were passed through centrifuge columns and F_1 -bound ^{32}P was collected directly into perchloric acid solution. Total bound ^{32}P (\circ , \bullet), bound $[\gamma\text{-}^{32}\text{P}]\text{ATP}$ (Δ , \blacktriangle) and bound $^{32}\text{P}_i$ (\square , \blacksquare) were determined.

that the conserved sequence may form at least part of the nucleotide-binding sites in the β -subunit and p21 *ras* protein. The side chain volumes of Gly, Ala and Val residues are 66.1 , 91.5 and 141.7 \AA^3 [28], respectively, and thus replacement of Gly or Ala by Val may change the structure of the ATP-binding site, possibly the orientation of the Lys-16 (p21 *ras* protein) or Lys-157 β residue.

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