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E. coli F_1-ATPase: site-directed mutagenesis of the β-subunit

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Residues βGlu-181 and βGlu-192 of *E. coli* F_1-ATPase (the DCCD-reactive residues) were mutated to Gln. Purified βGln-181 F_1 showed 7-fold impairment of 'unisite' P_i formation from ATP and a large decrease in affinity for ATP. Thus the β-181 carboxyl group in normal F_1 significantly contributes to catalytic site properties. Also, positive catalytic site cooperativity was attenuated from 5×10^4- to 548-fold in βGln-181 F_1. In contrast, purified βGln-192 F_1 showed only 6-fold reduction in 'multisite' ATPase activity. Residues βGly-149 and βGly-154 were mutated to Ile singly and in combination. These mutations, affecting residues which are strongly conserved in nucleotide-binding proteins, were chosen to hinder conformational motion in a putative 'flexible loop' in β-subunit. Impairment of purified F_1-ATPase ranged from 5 to 61%, with the double mutant F_1 less impaired than either single mutant. F_1 preparations containing βIle-154 showed 2-fold activation after release from membranes, suggesting association with F_0 restrained turnover on F_1 in these mutants.

F_1-ATPase; Mutagenesis; DCCD-reactive glutamate; Conserved glycine residue

1. INTRODUCTION

F_1-ATPases from various sources are inactivated by DCCD, which reacts specifically either with residue Glu-192 (*E. coli*, beef heart mitochondrial enzyme [1,2]) or residue Glu-181 (PS3 enzyme [3]) in the F_1 β-subunit. These glutamates are conserved in all species for which the F_1-β sequence is known [4–6] and studies of DCCD-inactivated enzyme have implicated them in different aspects of catalysis [7–9]. Mutagenesis experiments have suggested that substitution of Gln for Glu at β-181 or β-192 results either in total inactivation of ATPase (PS3 enzyme [10]) or partial inhibition of ATPase (*E. coli* [11]). However, purified F_1-ATPase was not studied in either report. Here we describe properties of purified *E. coli* F_1-ATPase containing βGln-181 or βGln-192 mutations.

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Abbreviations: DCCD, dicyclohexylcarbodiimide; residue numbers are rationalized to the *E. coli* β-subunit sequence throughout

There is a highly conserved sequence Gly-Gly-Ala-Gly-Val-Gly-Lys at residues 149–155 of F_1 β-subunits, which has been postulated to form a flexible loop that interacts with bound nucleotide phosphate groups in the catalytic site of F_1-ATPases [12,13]. Support for this postulate has come from nucleotide-binding studies with a 50-residue synthetic peptide corresponding to residues 133–182 of the β-subunit [14] and from mutagenesis of residue βLys-155 [15]. One of the interesting speculations regarding this putative 'loop' is that it may undergo substantial conformational displacement on substrate binding, as in adenylate kinase [16,17]. We introduced Gly⟶Ile substitutions at residues 149 and 154 of *E. coli* F_1 β-subunit to attempt to hinder conformational changes. Here we report the properties of purified *E. coli* F_1-ATPases containing the mutations βIle-149, βIle-154 and βIle-149, Ile-154 (double mutant).

2. MATERIALS AND METHODS

Generation of the mutants βGln-181, βGln-192, βIle-149 and βIle-154 has been described [11,18]. The double mutant

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mutant βIle-149,Ile-154 was generated from the βIle-149 mutant using the oligonucleotide used previously to make the βIle-154 mutant. A 200 bp *SalI-SstI* fragment from the M13 phage containing the desired two mutations was moved into plasmid pDP31 (which expresses the normal β-subunit, see [18]) to create plasmid pDP44 (expressing the double mutant β-subunit). DNA sequencing confirmed that there were no unwanted mutations in the *SalI-SstI* fragment. Plasmid pDP44 was used to transform strain AN1272 (which does not express the β-subunit) in order to study the effects of the mutations [18].

Methods for preparation of cell membranes and purified soluble F_1-ATPase, assays of ATPase, pH-gradient formation in membrane vesicles and growth yields on limiting glucose were as in [18]. Methods for kinetic assays and ligand binding by purified F_1-ATPase were according to [19]. Inactivation of purified F_1-ATPase by 25 μM DCCD was as in [20].

3. RESULTS AND DISCUSSION

3.1. *Purified F_1-ATPases from βGln-181 and βGln-192 mutants*

Both enzymes showed normal profiles for relative ATPase activity over the pH range 7.3–9.5 and had normal molecular size during gel filtration and normal subunit composition on SDS gels. Therefore, neither mutation caused a gross structural defect. Table 1 lists the specific ATPase activity of each purified enzyme and it is clear that, as we predicted from previous studies [11], although the βGln-181 mutation is much more detrimental for catalysis than the βGln-192 mutation, both mutants retain partial activity. The βGln-181 enzyme was just as sensitive to inactivation by DCCD as normal enzyme with $t_{1/2}$ = 1.2 min. In contrast, the βGln-192 enzyme was relatively insensitive to DCCD, with $t_{1/2} \geq 30$ min. Therefore, it does not appear that DCCD can inactivate the *E. coli* enzyme by reacting with residue βGlu-181 when residue Glu-192 is mutated to Gln.

Kinetic properties of the βGln-181 mutant enzyme were studied under 'unisite' conditions. In these procedures, substoichiometric amounts of ATP are incubated with F_1. Normal F_1 binds the ATP avidly at a 'first' catalytic site, hydrolyzes it but releases the resultant P_i slowly, and maintains a bound ATP/P_i ratio during hydrolysis of approximately unity. From fig.1 it is clear that the rate of association of ATP with F_1 (k_{+1}) and the initial rate of P_i formation were much slower than normal in the βGln-181 F_1 (see table 1, rows 1,2). In further experiments it was shown that other

Table 1

ATPase activity in membrane vesicles and in purified soluble F_1

Genotype	Membrane ATPase[a] (% of normal) (1)	Purified F_1-ATPase	
		Specific activity[b] (2)	% of normal (3)
1. Normal[c]	100	24	100
2. βGln-181	4[d]	0.26	1.1
3. βGln-192	22[d]	4.1	17
4. βIle-149	76[e]	18.3	76
5. βIle-154	17[e]	9.4	39
6. βIle-149,Ile-154	48	22.8	95

[a] All membrane ATPase activates were >80% sensitive to DCCD

[b] ATP hydrolyzed (μmol/min per mg) at 30°C, pH 8.5

[c] In normal enzyme the residues are Gly-149, Gly-154, Glu-181, Glu-192

[d] From [11]

[e] From [18]

properties of the high-affinity catalytic site were also abnormal in the mutant enzyme (table 2, rows 3–6). Particularly, the K_d for MgATP was 8.7×10^{-7} M as opposed to $\leq 7 \times 10^{-10}$ M in normal enzyme. K_d(MgADP) was found to be around 4 μM in both mutant and normal enzymes in separate experiments using [^{3}H]ADP and the centrifuge-column procedure.

Previous work [7] had shown that reaction of DCCD with PS3 F_1 inhibits ATP formation from bound ADP and P_i at a single catalytic site. From the results described here it is clear that the mutation βGlu-181 ⟶ Gln significantly changes intrinsic properties of the first catalytic site in F_1-ATPase, suggesting that the βGlu-181 carboxyl side chain is important in catalysis. Data presented here (pH profile, specific activity) render unlikely the possibilities that βGlu-181 acts as a general acid-base catalyst or interacts with substrates in a protonation/deprotonation-linked mechanism. Complicating the issue of the role in catalysis of residue βGlu-181, however, is the fact that the βGln-181 mutation also affected positive cooperativity between catalytic sites on F_1 (table 2, rows 7,8). The major determinant of the final level of catalytic activity was the degree to which positive cooperativity was attenuated. We have now found this to be the case in eight different F_1 β-subunit mutants for which detailed kinetic studies have been carried out in our laboratory

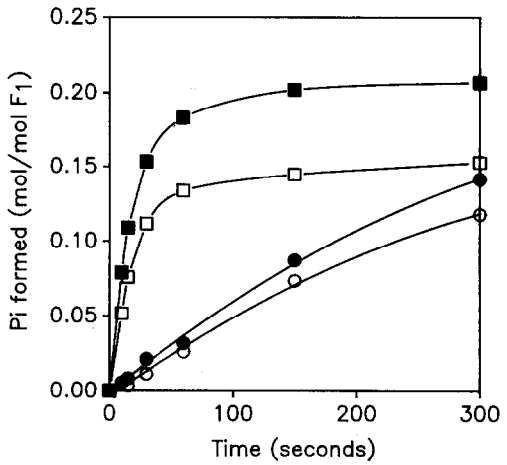

Fig.1. Unisite hydrolysis of [γ-^{32}P]ATP by normal and βGln-181 mutant purified F_1. The experiment was performed as in [19]. Briefly 0.3 μM [γ-^{32}P]ATP was mixed manually at 22°C with 1 μM F_1 in 50 mM Tris-SO_4, pH 8.5, 0.5 mM $MgSO_4$, 1 mM KH_2PO_4. Samples were directed quenched in $HClO_4$ at the times shown (○, □) or mixed with 3 × 10^5-fold excess unlabelled ATP then quenched 2 min later (●, ■). (Circles) βGln-181, (squares) normal F_1.

Table 2

Properties of purified βGln-181 mutant F_1-ATPase

	Normal (βGlu-181) (1)	Mutant (βGln-181) (2)
1. Initial rate of P_i formation[a] (mol P_i/mol F_1 per s)	1.75×10^{-2}	2.54×10^{-3}
2. k_{+1} ($M^{-1} \cdot s^{-1}$)[b]	$\geq 1 \times 10^5$	3×10^3
3. k_{-1} (s^{-1})[c]	7×10^{-5}	2.6×10^{-3}
4. K_d(ATP) ($= k_{-1}/k_{+1}$) (M)	$\leq 7 \times 10^{-10}$	8.7×10^{-7}
5. Bound ATP/bound P_i ratio[d]	1	<0.5
6. k_{+3}[e]	1.2×10^{-3}	8.4×10^{-4}
7. Multisite turnover rate (s^{-1})[f]	58	0.46
8. Promotion of catalysis[g]	5×10^4-fold	548-fold

[a] Measured as total P_i formed (bound plus free) after acid quenching of reaction mixture containing 0.3 μM ATP, 1 μM F_1 (see fig.1)

[b] Association rate constant for binding of ATP to first catalytic site

[c] Dissociation rate constant for release of ATP from first catalytic site

[d] Ratio of bound ATP/bound P_i approached equilibrium much more slowly than normal in the βGln-181 enzyme

[e] Rate of release of P_i from catalytic site under unisite conditions

[f] Measured rate of ATPase using same buffer and temperature as in unisite assays, but with saturating MgATP

[g] Calculated as multisite turnover rate divided by k_{+3} value (see [19])

([15,19,21,22] and this work). In all eight cases, the K_d(ATP) at the first catalytic site was found to be increased. Therefore, a major goal of future work will be to develop assays using monomeric isolated β-subunit which can effectively discriminate between direct effects of mutations on the catalytic site and effects on positive catalytic site-site cooperativity.

We did not perform detailed kinetic analyses of the βGln-192 purified F_1 since its specific ATPase activity was 17% of normal. We would predict the effect of this mutation is to cause a 6-fold decrease in positive catalytic site cooperativity without significantly changing unisite properties, as with the *uncD412* mutation [19]. This would be consistent with conclusions arrived at by others from the DCCD reaction of residue βGlu-192 in F_1-ATPase [8,9]. As we pointed out previously, covalent reaction of DCCD with residue βGlu-192 has a much more drastic effect on ATPase activity than does mutation of βGlu-192 to Gln [11].

3.2. *Effect of the double mutation βIle-149,Ile-154 on cell growth and membrane properties*

We found previously that each of the single mutations βGly-149⟶Ile or βGly-154⟶Ile reduced growth yield of cells on limiting glucose [18]. We found here that in this assay the double mutant cells grew slightly better than either single mutant. In the assay of pH-gradient formation in membrane vesicles, NADH caused >90% of quenching of acridine orange fluorescence in all three cases. The values for ATP-induced quenching were: βIle-149, 78%; βIle-154, 47%; βIle-149,Ile-154, 61%; normal membranes, 87%; membranes lacking F_1, 0%. Membrane ATPase activities for all three mutants are shown in table 1. Because the growth yield, membrane ATPase and ATP-induced pH gradient formation seemed higher than expected in the double mutant we checked to be sure that the double mutation on plasmid pDP44 had not reverted during cell growth; DNA sequencing confirmed that both Gly⟶Ile mutations were still present. Thus, the surprising conclusion was that the double mutation did not seriously impair cell growth, membrane ATPase or ATP-driven proton pumping, indeed it relieved the effects of the βIle-154 single mutation to a certain degree.

3.3. *Purified F_1-ATPase from the βIle-149, βIle-154 and βIle-149,Ile-154 mutants*

Each of these enzymes showed normal molecular size (Sephacryl S-300 gel filtration), subunit composition (SDS-gels) and pH-dependent ATPase profile. The specific ATPase activities of the three mutant enzymes are shown in table 1, column 3 and in no case was activity seriously impaired. This result was unexpected for several reasons. First, residues Gly-149 and Gly-154 are highly conserved in a wide range of nucleotide-binding proteins. Second, it may be anticipated [23] that the substitution of Ile for Gly (relative bulk = 169 $Å^3$ vs 66 $Å^3$, [24]) in positions at the ends of the putative 'flexible loop' in F_1-β would have significant effects. Third, it should be noted that the mutations βAla-151⟶Val and βLys-155⟶Gln have been shown to impair significantly enzyme activity [15,25].

The results were unusual in two other respects. First, the specific activity of the double mutant enzyme was higher than that of either single mutant enzyme. The effects of the two mutations were not additive, rather they seemed anti-cooperative. Second, there was apparent activation of both the βIle-154 and βIle-149,Ile-154 enzymes on release from membranes (cf. table 1, columns 1,3). Immunoblot experiments on membranes from each of the mutant strains, using a combination of anti-β and anti-b antibodies, showed that the amount of F_1F_0 enzyme in the membranes was similar to normal in all three mutants. In previous studies of β-subunit mutant enzymes we have found that membrane ATPase activity generally correlates fairly well with purified F_1-ATPase specific activity when expressed as % of normal (isogenic) activity, the only exceptions being when the mutation impairs activity strongly such that the resultant low membrane ATPase is difficult to measure accurately over background. The unusual activation of F_1-ATPase from the two mutant enzymes containing Ile at residue β-154 after release from membranes suggests that a membrane-associated restraint on turnover rate had been relieved. Therefore further study of this region of sequence in relation to conformational movement does appear to be justified. However, the present results show that use of a selection procedure in conjunction with directed or localized mutagenesis of this region of sequence is advisable.

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