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Identification of a mutation in *Escherichia coli* F_1-ATPase β-subunit conferring resistance to aurovertin

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A mutation conferring aurovertin resistance on *Escherichia coli* F_1-ATPase was identified as R398→H in the F_1 β-subunit. β-subunit from the mutant does not bind aurovertin; therefore our results suggest the region of sequence around residue β-398 is involved in aurovertin binding. Since nucleotide and aurovertin binding to isolated β-subunit are not mutually exclusive, the data further suggest that the β-subunit catalytic nucleotide-binding domain does not include residue 398. The mutation prevented aurovertin inhibition of ATPase at pH 6 and 8.5, implying charge on the arginine side-chain is not a major determinant of aurovertin binding or that the pK of R398 is shifted due to a peculiar environment. The equivalent residue is usually arginine in F_1 β-subunits of different species; notably in the aurovertin-insensitive thermophilic bacterium PS3 F_1-ATPase, this residue is phenylalanine.

Aurovertin resistance; F_1-ATPase β-subunit; Mutation

1. INTRODUCTION

Aurovertin is an antibiotic that inhibits oxidative phosphorylation by binding tightly and specifically to the β-subunit of F_1-ATPases [1,2]. In *Escherichia coli* aurovertin inhibits growth of cells on nonfermentable substrates [3], it inhibits the activity of purified soluble F_1 with 50% inhibition occurring at ~1 μM, and it binds with K_d 3–6 μM to isolated F_1 β-subunit [4,5].

Aurovertin has proved to be a useful probe of conformational changes and catalytic mechanism of F_1-ATPases, for the reason that it fluoresces when bound to the enzyme. Chang and Penefsky [6] used the antibiotic to demonstrate respiration-induced conformational changes of F_1-ATPase in submitochondrial particles, experiments which we can now interpret as a demonstration of transmembrane $\Delta\mu H^+$-induced conformational change of the aurovertin-binding site on β-subunit of F_1-ATPase. Later, Wise and colleagues [7] used aurovertin-fluorescence measurements to demonstrate that some mutations in α-subunit of *E. coli* F_1-ATPase block α/β-intersubunit conformational interactions. They proposed that such interactions were an integral part of the catalytic mechanism. Several other important studies utilizing aurovertin as a fluorescent probe could be cited (e.g. [8,9]).

It would therefore be valuable to know where the binding site for aurovertin is on F_1 β-subunit. Satre et al. [3,10] isolated aurovertin-resistant mutants of *E. coli* and demonstrated that the mutations mapped in the region of the *unc* operon. One of the mutations (in strain MA12) was examined in some detail and shown to confer aurovertin resistance to F_1-ATPase. Further, aurovertin resistance was shown to be due to a mutation in F_1 β-subunit by in vitro subunit dissociation-reassociation experiments, and isolated β-subunit from MA12 was shown not to bind aurovertin [4]. Here we have identified the mutation in MA12 β-subunit.

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2. MATERIALS AND METHODS

2.1. *General*

All molecular biology methods were as previously described [11,12] except where noted below. Strains of *E. coli* were as follows: MA12, aurovertin-resistant mutant strain [3,10]; AN818 [11], JM109 [13], AN1339 [14]. Plasmids used were pUC118 [15] and pDP31 [12]. For membrane preparations [16], cells were grown 6 h in 50 ml of glucose/minimal salts/2.5% LB/ampicillin then inoculated into 1 l glucose/minimal salts/25% LB/ampicillin. ATPase was measured as described by Wise et al. [7].

2.2. *Cloning and sequencing of the* uncD *gene from strain MA12*

Chromosomal DNA from strain MA12 was prepared, digested with *Pst*I and run on agarose gels. Slices corresponding to predicted DNA fragment sizes of 0.94 and 1.2 kb were excised and the fragments were ligated into *Pst*I-cut pUC118. From the restriction map of the *unc* operon, we expected to obtain recombinants containing the promoter-proximal and -distal halves of the *uncD* (β-subunit) gene. The ligation mixture was transformed into strain JM109 on LB/ampicillin/Xgal/IPTG plates, and colorless transformants were grown up in LB/ampicillin. Dot hybridization was then carried out on nitrocellulose using DNA from 50 μl alkali-lysed cells. The ^{35}S-labeled probe was a fragment containing the *uncD* (β-subunit) gene, labeled by the random-primed method [17]. For confirmation that the desired clones were identified, restriction digestions were carried out on plasmid minipreps. Double-stranded DNA sequencing was carried out by the chain termination method using a series of oligonucleotide 20-mers covering the *uncD* gene.

2.3. *Construction of a plasmid for expression of aurovertin resistance*

Plasmid pMA12.A (pUC118 containing the 1.2 kb *Pst*I fragment corresponding to the promoter-distal half of the *uncD* gene with the aurovertin resistance mutation in it) was digested with *Xma*III and *Kpn*I and the resultant 1 kb fragment was isolated from an agarose gel. Plasmid pDP31 was digested with *Hin*dIII-*Xma*III yielding a 1.4 kb fragment containing the normal promoter-proximal half of *uncD*. Both fragments were mixed with *Hin*dIII/*Kpn*I-digested pUC118, ligated, and transformed into JM109 on LB/ampicillin/Xgal/IPTG plates. Plasmids were prepared from colorless transformants, checked by restriction digestion and sequenced to confirm that the aurovertin-resistance mutation was present. One plasmid containing the mutation was named pMA12.R.

3. RESULTS

The entire *uncD* (β-subunit) gene from strain MA12 was sequenced and was found to contain only a single base change. Codon 399 (normally CGC) was changed to CAC, resulting in the amino acid change R398 → H.

In order to confirm that this mutation was responsible for aurovertin resistance, we moved the mutation from the initial clone (pMA12.A) into plasmid pDP31 which expresses the normal *uncD* gene [12], yielding plasmid pMA12.R as described in section 2. We transformed strain AN818 with both pMA12.R and pDP31. Strain AN818 (*recA*), carrying the mutation G214R in β-subunit, is completely defective in F_1-ATPase assembly and shows zero membrane-bound ATPase activity [11,16]. For further comparison we also used strain MA12 and the haploid normal strain AN1339. The results of membrane ATPase and growth assays of these four strains are shown in table 1. The mutation β-R398H, when expressed from plasmid pMA12.R, gave small reduction of growth yield on limiting glucose and also of growth on succinate plates. Membrane ATPase ac-

Table 1

Membrane ATPase and growth characteristics

Strain	Genotype	Membrane-ATPase (μmol/min per mg)	Growth yield on limiting glucose[a] (OD_{590})	Growth on succinate[b]
pDP31/AN818	*unc*$^+$	2.4	0.78	+ + +
pMA12.R/AN818	β-R398H	1.7	0.72	+ +
pUC118/AN818	*unc*$^-$	0	0.42	−
MA12	β-R398H	N.D.[c]	0.89	+ + +
AN1339	*unc*$^+$	N.D.[c]	0.88	+ + +

[a] Growth yield on 3 mM glucose. Ampicillin (50 μg/ml) was included in medium for plasmid-bearing strains

[b] Succinate plates

[c] N.D., not determined

Table 2

Inhibition of membrane ATPase by aurovertin

Strain	% ATPase activity			
	pH 6.0[a]		pH 8.5[a]	
	No aurovertin	10 μM aurovertin	No aurovertin	10 μM aurovertin
pDP31/AN818	100	18	100	5
pMA12.R/AN818	100	90	100	98

[a] The pH 6.0 assay done in 50 mM succinate-Tris buffer; the pH 8.5 assay done in 50 mM Tris-SO_4 buffer

tivity was slightly-reduced as compared to the control (pDP31/AN818). Thus, under these conditions the mutation conferred a slight Unc phenotype, although this was not evident in the haploid strain MA12.

Table 2 shows membrane ATPase assays in the presence and absence of aurovertin. From the data it is evident that the mutation R398H β-subunit is sufficient to confer aurovertin resistance on membrane-bound F_1-ATPase. When aurovertin inhibition of membrane ATPase was measured at pH 6.0 vs pH 8.5 (table 2), little difference was seen in the mutant. Consistent with previous data on inhibition of cell growth at different pH [3], aurovertin was slightly less inhibitory toward normal F_1-ATPase at the lower pH.

4. DISCUSSION

Our results suggest that residue R398 is part of the aurovertin-binding site in the normal F_1 β-subunit. The mutation R398H prevents aurovertin binding to the β-subunit and F_1-ATPase [3,4,10], but does not significantly affect F_1-ATPase activity, oxidative phosphorylation, or the ability of β-subunit to reconstitute with normal α-, γ- and ε-subunits to form an active ATPase [3,10].

If the charge of residue R398 were important in aurovertin binding, the replacement of arginine by histidine might have been expected to prevent binding at pH 8.5, but to be less effective at pH 6.0. This was not the case. Thus, other factors such as bulk, stereochemistry or local environment changes might be invoked to explain the effect of the mutation. One possibility is that the R398 pK is shifted toward a less alkaline value due to a peculiar amino acid environment. This explanation is plausible as R398 is close to two other arginine residues, R392 and R394.

Our results also have important consequences for the location of the catalytic nucleotide-binding domain in *E. coli* F_1 β-subunit. It has been demonstrated that binding of ADP and aurovertin or of ATP and aurovertin may occur simultaneously to the isolated β-subunit of *E. coli* [4,18]. Also it is known that aurovertin inhibits *E. coli* F_1 in an uncompetitive manner [19]. Previously it has been suggested [20] that the catalytic nucleotide-binding domain of β-subunit extends from residues 140–335 approximately. The exact limits of the sequence encompassing this domain are, of course, speculative. The data presented here show that the region of sequence around residue 398 cannot be part of the nucleotide-binding domain.

Finally it is interesting to note that the residue equivalent to *E. coli* F_1 β-398 is usually, but not always, arginine in other species. Out of 21 species for which the sequence is currently known, arginine is present at this position in 18. Notably, this residue is phenylalanine in thermophilic bacterium PS3 [21] and PS3 F_1 is known to be aurovertin insensitive [22].

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