Mutation of Alanine 24 to Serine in Subunit c of the Escherichia coli
$F_1F_0$-ATP Synthase Reduces Reactivity of Aspartyl 61 with
Dicyclohexylcarbodiimide*

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Dicyclohexylcarbodiimide (DCCD) inhibits the activity of the $F_1F_0$-H$^+$ ATP synthase of Escherichia coli by reacting with aspartyl 61 in subunit c of the $F_0$ sector to form a stable N-acylurea. The segment of chromosomal DNA which codes the subunits of the $F_0$ was cloned from four independently isolated DCCD-resistant mutants, and the sequence of the subunit c gene (uncE) was determined. An Ala$^{24}$ to serine (A24S) substitution was found in the subunit c gene of each mutant. The A24S uncE gene was cloned into the BamHI site of a mutant derivative of plasmid pBR322. The A24S subunit c conferred DCCD resistance to a variety of recipient E. coli strains when it was over-expressed from this plasmid. A 7-base pair deletion beginning at position 132 of the plasmid vector was responsible for the observed overexpression. Hoppe et al. (Hoppe, J., Schairer, H. U., and Sebald, W., 1980, Eur. J. Biochem. 112, 17-24) had previously shown that mutation of subunit c Ile$^{28}$ to threonine or valine resulted in DCCD resistance. The DCCD sensitivities of the membrane ATPase of these mutants and the A24S mutant were compared. DCCD sensitivity decreased in the order: wild-type $\approx$ I27V $\approx$ I28T $\approx$ A24S. The venturicidin sensitivities of wild-type and mutant membranes were also examined. The membrane ATPase of the I28T and I28V mutants was venturicidin resistant whereas the A24S substitution resulted in a hypersensitivity to inhibition by venturicidin. These results support a model in which subunit c folds in the membrane like a hairpin, where the region of residues 24-28 in transmembrane helix-1 is close to that of aspartyl 61 in transmembrane helix-2.

A membrane-associated, H$^+$-transporting ATP synthase catalyzes the synthesis of ATP during oxidative phosphorylation in Escherichia coli and other eubacteria. Structurally similar ATP synthases are found in mitochondria and chloroplasts. These enzymes are composed of two sectors termed $F_1$ and $F_0$. The $F_1$ sector is easily released from the membrane and, in soluble form, catalyzes ATP hydrolysis. The $F_0$ sector extends through the membrane and catalyzes proton translocation through the membrane. When the two sectors are coupled, the enzyme functions as a reversible, H$^+$-transporting ATPase or ATP synthase (Senior, 1988; Fillingame, 1990). The subunit composition of $F_1$ and $F_0$ in E. coli appears to be the simplest found in nature (Senior, 1988) wherein $F_1$ is composed of five subunits and $F_0$ of three subunit types in a $a_3b_3c_1$ stoichiometry (Foster and Fillingame, 1982).

The membrane sector of mitochondria was originally named "$F_0$" (where the letter O subscript indicates oligomycin) to designate a detergent-solubilized preparation that restored oligomycin-sensitive ATPase activity when reconstituted with purified $F_1$ (Racker, 1976). Dicyclohexylcarbodiimide (DCCD)$^1$ also inhibits the ATPase activity of $F_0$, by covalently binding to a site in $F_0$ (Beechey et al., 1967). DCCD binds covalently as the N-acylurea adduct of aspartyl 61 in a small, chloroform-methanol soluble protein of $F_0$ termed proteolipid or subunit c (Cattell et al., 1971; Fillingame, 1975; Sebald and Hoppe, 1981). The isolation and analysis of DCCD-resistant mutants of E. coli helped to establish that ATPase inhibition was caused by the specific reaction of DCCD with subunit c (Fillingame, 1975, 1980; Friedl et al., 1977). Hoppe et al. (1980) sequenced the subunit c of six of the mutants isolated by Friedl et al. (1977) and found an Ile$^{28}$ to valine or threonine substitution in each mutant. To rationalize the effects of these substitutions on DCCD reactivity, subunit c was proposed to fold like a hairpin such that Ile$^{28}$ neighbors the DCCD-reactive Asp$^{61}$ in the membrane (Hoppe et al., 1980; Sebald and Hoppe, 1981; Fillingame, 1990). The amino acid substitutions in the four independently isolated DCCD-resistant mutants described by Fillingame (1975, 1979) had not been examined. We report here that all four mutants contain Ala$^{24}$ $\rightarrow$ Ser substitutions in subunit c. These substitutions support a hairpin model for subunit c in which both Ile$^{28}$ and Ala$^{24}$ must lie close to Asp$^{61}$ in the membrane.

**EXPERIMENTAL PROCEDURES**

Strains of E. coli—Generalized transductions were carried out with plasmid P1 cm1100 as described by Miller (1972). Most of the strains used are isogenic $\text{Ilv}^+$/unc cotransductants of strain AN346 (F, $\text{argH}^+$, $\text{serL}^+$, $\text{met}^+$, $\text{hcn}^+$, $\text{Iac}^+$, $\text{EcoRI}$). These transductants include strain MM128 [uncE101 (A24S)] and strains carrying the uncE102 (A24S), uncE103 (A24S) and uncE104 (A24S) alleles (Fillingame, 1979). Some strains were made recA56 by plasmid P1 transduction as described by Mosher et al. (1983). The recA56 strains include: MM383 [uncM], MM441 [uncE107 (D61N)], and MM394 [uncE114 (Q42E)]. Strain MM349 [uncB402] is a derivative of strain R1 [F $\cdot$ pro, leu, thi, lacY, rpsL, hsdR2 (r, m)]; Bolivar and Backman, 1979] that was made recA56

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††The abbreviations used are: DCCD, dicyclohexylcarbodiimide; HPLC, high performance liquid chromatography; Hepes, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid.

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by Dr. Mary Mosher in this laboratory. Strains DC13 (128T) and DC25 (128V), described in Hoppe et al. (1980), are Inv+ Met+ derivatives of E. coli strain Ymel (Δ [F-, lacI, fadR, but12, rha]) and were a gift of Dr. John E. G. McCarthy (Institut für Biotechnologie, Braunschweig, Federal Republic of Germany).

Genetic Methods—Minimal medium 63L, LB medium, and 2YT medium are described in Miller (1972). M63 minimal medium agar plates were supplemented with 2 μg/ml thiamine hydrochloride, 0.2 mM uracil, 0.2 mM L-arginine, and 40 μM 2,3 dihydrobenzoic acid as required, and 0.2% glucose was used as a carbon source. A combination of 0.6% disodium succinate hexahydrate, 0.2% potassium acetate, and 0.2% potassium lactate was used as an alternative carbon source to glucose in plates used for complementation analysis or in the analysis of DCCD resistance. DCCD was added to the succinate-acetate-lactate minimal agar medium at 55 °C in 0.01 volume of ethanol at a final concentration of 5 mM (Fillingame, 1975).

Cells for a biochemical characterization were grown on liquid M63 medium supplemented with 0.2 mM L-arginine, 40 μM 2,3 dihydrobenzoic acid as required, 0.6% disodium succinate hexahydrate, 0.2% potassium acetate, and 0.2% potassium lactate.

Results

Localization of Mutations Conferring DCCD Resistance—Chromosomal DNA was isolated from four independently selected DCCD-resistant mutants and the DNA digested with HindIII. The HindIII fragment of chromosomal DNA containing the uncBEFHA genes (bases 870–5308) was cloned into the HindIII site of plasmid pBR322. Recombinant plasmid transformants containing the wild-type uncB gene were selected based upon complementation of the uncBEFHA genes (bases 870–5308) and treated with 0.01 ml of DCCD in ethanol for 20 min at 30 °C. The ATPase assay was then initiated by the addition of 0.1 ml of 4 mM [γ-32P]ATP.

To prove that the Ala24 → Ser substitution was the mutation causing DCCD resistance, the BamHI(1727) to HpaI(2162) DNA fragment containing uncE was cloned from the plasmids described above into the BamHI and HindIII sites of phage M13mp18 and the uncE DNA sequence. Each of the four cloned uncE genes showed a CGT(Ala) to TCT(Ser) change in the codon for residue 24 of subunit c and no other changes elsewhere in the coding sequence of the protein.

To prove that the Ala24 → Ser substitution was the mutation causing DCCD resistance, the BamHI(1727) to HpaI(2162) DNA fragment of uncE101(A24S) DNA was subcloned from the larger plasmid described above into the BamHI and PvuII sites of plasmid pBR322. This plasmid (pM0101) only weakly complemented uncE recipient strains but did not confer DCCD-resistant growth. In contrast, plasmid pCP35 (Fillingame et al., 1984), which was thought to be equivalent to plasmid pM0101 but carries the wild-type uncE gene, gave robust complementation with the same uncE recipient strains. A control plasmid was constructed in which the wild-type BamHI to HpaI DNA fragment was cloned into the BamHI and PvuII sites of plasmid pBR322, and, in contrast to plasmid pCP35, this plasmid (pM0100) gave weak complementation of uncE recipient strains. To determine whether the vector DNA was responsible for the differences in complementation, segments of the pCP35 and pM0101 plasmids were exchanged. The plasmids were cut with BamHI and at the NdeI site (position 2296) in the pBR322 vector DNA (Fig. 1). The 655-base pair BamHI/NdeI fragment containing the uncE gene was separated from the 2449-base pair BamHI/NdeI vector fragment by agarose gel electrophoresis, and the purified fragments were mixed and ligated. The product plasmid pM0351 contains the uncE101 (A24S) gene in the pCP35 BamHI/NdeI vector DNA. Plasmid pM0351 gave robust complementation when transformed into several uncE mutants (Table I) and conferred DCCD-resistant growth to both mutant and wild-type transformants. On the other hand, the plasmid containing the wild-type uncE gene in the pM0101-derived vector DNA complemented poorly and confirmed that the differences in complementation efficacy were caused by the vector DNA.

The EcoRI (4361) to BamHI (375) segment of pCP35 vector DNA was sequenced. When compared with pBR322 DNA, the pCP35-derived DNA was found to have a 7-base pair deletion of nucleotides (T1295GGATGTC(T)1302). It is unclear from the DNA sequence whether T132 or T139 was deleted. The

![FIG. 1. Plasmid pCP35 and related plasmids.](image-url)
**DCCD-resistant mutants of F,F-ATP Synthase**

### Table I

| Plasmid        | uncE allele | Vector* | Wild type | Growth of transformants on succinate |
|----------------|-------------|---------|-----------|-------------------------------------|
| pMO100         | W.T.        | pBR322  | +         | ++ + + + + + + + + + + + + + + + + |
| pMO101         | E101        | pBR322  | +         | + + + + + + + + + + + + + + + + |
| pCP5          | W.T.        | pCP5    | +         | + + + + + + + + + + + + + + + + |
| pMO351        | E101        | pCP5    | +         | + + + + + + + + + + + + + + + + |

* BamHI(1727) → HpaI(2162) fragment of unc DNA. W.T., wild type; E101, A245.

**Efficiency of Complementation Related to Expression of Plasmid-coded Subunit c**—It seemed possible that the difference in the complementation efficacy of the plasmids shown in Table I might result from differences in level of expression of subunit c. The amount of subunit c in membranes of plasmid-containing cells was compared. For this purpose, strain MM994, which carries the uncE114 (Q42E) mutation, was transformed with plasmid pMO101 (uncE101, A245) or plasmid pMO351 (uncE101, A245) so that the amounts of chromosomal versus plasmid-borne products could be compared. The Q42E mutation results in a charge difference that permits separation of the two proteins by anion exchange HPLC (Hermolin and Fillingame, 1989). When pMO101 and pMO351 transformant colonies of strain MM994 [uncE114 (Q42E)] were compared on 2YT-ampicillin-rich medium, the pMO101 transformants were distinctly smaller. In the process of growing cells to do this experiment, we noticed that cells from cultures of strain pMO101/MM994 eventually formed a mixture of large and small colonies on LB-ampicillin or 2YT-ampicillin plates. When a small single colony from an LB-ampicillin plate was grown in 5 ml of LB-ampicillin medium overnight and 2.4 ml of that culture used to inoculate 1200 ml of LB-ampicillin medium, the product cells formed small and large colonies in approximately a 50/50 ratio. A more homogeneous population of small colony-forming cells was obtained by scraping two small colonies from an agar plate and directly inoculating 200 ml of LB-ampicillin medium. The yield of subunit c from these pMO101/MM994 cultures is compared with that from a pMO351/MM994 culture in Table II, and the proportions of plasmid-derived A24S protein to chromosomal-derived Q42E protein are indicated by the HPLC profiles of Fig. 2. In the culture producing uniformly small colonies, plasmid pMO101 generated less subunit c than that produced by the chromosomal gene coding.

### Table II

**Comparison of plasmid-derived yield of A24S subunit c from plasmid pMO351 and pMO101 in strain MM994 harboring Q42E chromosomal subunit c gene**

| Strain         | Colony size | Yield of subunit c | Ratio of subunit c |
|----------------|-------------|--------------------|--------------------|
|                |             | Total              | A24S               |
|                |             | Q42E (chromosomal) | (plasmid)          |
| pMO351/MM994  | 100% large  | 2.07               | 0.42               | 1.65               |
| pMO101/MM994  | 50% large   | 1.05               | 0.40               | 0.65               | 4.0 |
| pMO101/MM994  | 50% small   | 0.54               | 0.34               | 0.20               | 1.6 |
| pMO101/MM994  | >95% small  | 0.45               | 0.34               | 0.20               | 1.6 |

* Cells were grown on LB-ampicillin medium as described under “Results.” The culture of pMO101/MM994 which formed >95% small colonies was grown directly from small single colonies.

* Size of colonies produced on 2YT-ampicillin plates after growth of liquid culture for subunit c purification.

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![Fig. 2. Separation of A24S, plasmid-derived subunit c from Q42E, chromosomal-derived subunit c by HPLC anion exchange chromatography](image)

The purified subunit c fraction from A24S-producing plasmid transformants of strain MM994 (chromosomal Q42E) were separated on an AX300 anion exchange HPLC column by elution with an ammonium acetate gradient in chloroform-methanol-water (4:4:1) solvent. The conditions are given in Hermolin and Fillingame (1989). Trace 1, plasmid pMO351-transformed strain MM994; trace 2, plasmid pMO101-transformed strain MM994 from culture producing 50% large and 50% small colonies (see “Experimental Procedures”); trace 3, plasmid pMO101-transformed strain MM994 from culture producing >95% small colonies (see Table II).
the Q42E protein. Considerably more subunit c was produced by the culture of large and small colony-producing cells, and this suggests that mutation of the plasmid led to greater A24S subunit c production and more vigorous growth because of biochemical complementation of the nonfunctional Q42E subunit c protein. In these experiments, plasmid pMO351 produced four times as much subunit c as that produced by the chromosomal gene.

Mixing of A24S and Q42E Subunits in Fo, Restores DCCD Sensitivity to ATPase—The uncE114 (A24S) mutant shows DCCD-resistant ATPase activity because of reduced reactivity of DCCD with Asp \( \text{E}^{61} \) (Fillingame, 1975). The uncE114 (Q42E) mutant exhibits DCCD-resistant ATPase activity because of uncoupling of the Fo and F1 (Mosher et al., 1985). Membranes from uncE114 plasmid transformants of uncE114 cells showed an intermediate sensitivity to DCCD, i.e. greater inhibition was observed than with A24S mutant membranes but less inhibition than with wild-type membranes (Fig. 3). This intermediate sensitivity occurs with membranes containing one Q42E subunit/8-9 A24S subunits.

Comparison of DCCD Resistance Conferring by the A24S, I28T, and I28V Mutations—Hoppe et al. (1980) had previously reported DCCD-resistant mutants with substitutions at Ile \( \text{E}^{61} \) of subunit c. The relative DCCD sensitivity of the I28T, I28V, and A24S mutants was compared. The ATPase of I28T and A24S membranes was equally resistant to inhibition by DCCD (Fig. 4). The I28V membrane ATPase showed an intermediate sensitivity between that of the aforementioned mutants and that of wild type (Fig. 4). It is of interest that all three DCCD-resistant mutants had membrane ATPase activities that were elevated relative to wild type. An elevated membrane ATPase activity was also reported in previous studies of the A24S mutant (Fillingame, 1975; Fillingame and Wopat, 1978).

Venturicidin Resistance Is Conferred by the I28T and I28V Mutations but Not by the A24S Mutation—The venturicidin sensitivity of the ATPase of mutant and wild-type membrane is compared in Fig. 5. As originally demonstrated by Perlin et al. (1985), venturicidin is an effective inhibitor of the wild-type membrane ATPase. The I28T and I28V mutations resulted in venturicidin resistance, with the I28T membrane ATPase being most resistant. On the other hand, the A24S mutation led to a membrane ATPase that was hypersensitive to inhibition by venturicidin (Fig. 5).

DISCUSSION

The uncE genes of four independently isolated, DCCD-resistant mutants were sequenced in this study, and each of the four genes coded A24S substitutions in subunit c. In contrast, Hoppe et al. (1980) had sequenced the subunit c protein from six DCCD-resistant mutants, and all six proteins contained either 128V or 128T substitutions. The I28T and A24S mutation confer equivalent DCCD resistance as judged by growth of cells on succinate-acetate-malate in the presence of DCCD and by the resistance of the membrane ATPase. It is unclear why the two collections of mutants differ with respect to the mutated residue. The mutants isolated by Fillingame (1975, 1979) were selected on a combined succinate-acetate-malate carbon source on plates containing 5 mM DCCD. The cells were mutagenized with N-methyl-N'-nitro-nitroso-guanidine. The mutants isolated by Fried et al.
The finding that mutations in both Ala24 and Ile28 affect reactivity of DCCD with Asp61 most simply suggests a binding site for DCCD between residues 24 and 28 in the first transmembrane helix of subunit c. The binding site must obviously be close enough to Asp61 in transmembrane helix-2 for DCCD to react with the aspartyl β-carboxyl group. The evidence that subunit c does fold like a hairpin with two transmembrane helices has been reviewed elsewhere (Miller et al., 1990; Fillingame, 1990). The region surrounding residues 24–28 is highly conserved in all subunit c (see Fig. 6), and we have suggested that this region may interact with the region around Asp61 during the proton transfer cycle (Miller et al., 1990; Fillingame, 1990). The Gly23 and Gly27 residues of transmembrane helix-1 are absolutely conserved, and it is of interest that threonine or valine substitutions at position 24 will either have a deleterious effect on ATP synthase function or not confer DCCD resistance.

Interpretation of the carbodiimide inhibition experiments is complicated by the complexity of the reaction. DCCD must first react with the carboxylate form of Asp61 to yield on O-acetylurea and the adduct then rearrange to form the N-acetylurea product (Williams and Ibrahim, 1981):

\[ \text{R-CO}^- + H^+ + \text{DCCD} \rightarrow \text{O-acetylurea} \]  

\[ \text{O-acetylurea} \rightarrow \text{N-acetylurea} \]  

Reaction 1 might only occur after DCCD was bound at its binding site. Alternatively, reaction 1 might occur between an Asp61 carboxylate group that was exposed to the lipid bilayer and DCCD that was simply dissolved in that lipid bilayer. The carbodiimide binding site might then function in promoting the acyl migration occurring in reaction 2. Binding might simply prevent the competing hydrolysis reaction of the O-acetylurea to dicyclohexylurea that would regenerate a free carboxyl group.  

Perlin et al. (1985) have shown that venturicidin inhibits the activity of E. coli F$_{1}$F$_{0}$-ATPase and that its binding reduces the reaction of DCCD with subunit c. Similar results are seen with both venturicidin and oligomycin in mitochondria (Enns and Criddle, 1977; Kiehl and Hatefi, 1980), and the binding sites for all three compounds are thought to overlap. Oligomycin is a very weak inhibitor of the E. coli F$_{1}$F$_{0}$-ATPase (Perlin et al., 1985). A number of substitutions in the mitochondrial subunit c result in oligomycin and venturicidin resistance in yeast and Neurospora (Sebald and Hoppe, 1981; Galanis et al., 1989), and the sites leading to resistance overlap. Interestingly, venturicidin resistance in yeast results from mutations in residues corresponding to position 25, 27, and 29 of the E. coli protein (Galanis et al., 1989). The yeast venturicidin resistance site on transmembrane helix-1 thus surrounds the 128T venturicidin resistance site found in the E. coli protein. A second venturicidin resistance site in yeast lies in the equivalent of residues 58 and 59.
of E. coli subunit c. The binding site thus seems to span or lie between the two membrane-traversing helices. It will be of interest to see if mutation of residues causing venturicidin resistance in yeast will lead to a venturicidin-resistance for the E. coli F,Fo-ATPase.

Our unpublished NMR studies on isolated subunit c in chloroform-methanol solvent indicate an extended α-helix from Asp7 through Leu14. The structure of the purified subunit in chloroform-methanol solution must resemble that found in the membrane since the I28T mutation reduces the reactivity of Asp7 with DCCD even when the protein is purified. The conserved Gly23 and Gly27 residues would lie on one face of this α-helical structure and GlyAla25 and GlyAla29 on the opposite face of the same helix. The glycine residues are separated by bulky, hydrophobic residues and would form a screwlike groove around the helix of the protein. The groove could accommodate the packing of bulky side chains from another α-helix. During the course of proton translocation and the consequent conformational changes leading to ATP synthesis (Fillingame, 1989), one could easily envision a rotation of helices relative to each other. For example, in one state the Val80 and Met57 side chains of one subunit c could lie in the groove formed by Gly23 and Gly27 in the same, or a second, subunit c and in the alternate state rotate up the helix to the part of the groove formed by Ala25 and Gly29.

It is of interest that the substitutions at positions 24 and 28 which cause DCCD resistance also activate the ATPase activity of membrane-bound F, (Fig. 4). The small conformational changes that result from the DCCD resistance mutations in this region of subunit c must ultimately then promote conformational changes at the catalytic site of F,. Mutations in Asp25 on the opposite helix also alter the properties at the catalytic site (Fillingame et al., 1984). These changes in conformation may obviously be related to those required for the coupling of proton translocation to ATP synthesis.

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