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Mutation of Alanine 24 to Serine in Subunit *c* of the *Escherichia coli* F_1F_O-ATP Synthase Reduces Reactivity of Aspartyl 61 with Dicyclohexylcarbodiimide*

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Dicyclohexylcarbodiimide (DCCD) inhibits the activity of the F_1F_O-H^+ ATP synthase of *Eschiericia coli* by reacting with aspartyl 61 in subunit *c* of the F_O sector to form a stable *N*-acylurea. The segment of chromosomal DNA which codes the subunits of the F_O 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 *Bam*HI 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 overexpressed 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 ≫ I27V > I28T = 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_O. The F_1 sector is easily released from the membrane and, in soluble form, catalyzes ATP hydrolysis. The F_O 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_O in *E. coli* appears to be the simplest found in nature (Senior, 1988) wherein F_1 is composed of five subunits in an $\alpha_3\beta_3\gamma_1\delta_1\epsilon_1$ stoichiometry and F_O of three subunit types in a $a_1b_2c_{10}$ stoichiometry (Foster and Fillingame, 1982).

The membrane sector of mitochondria was originally named "F_O" (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_1F_O by covalently binding to a site in F_O (Beechey *et al.*, 1967). DCCD binds covalently as the *N*-acylurea adduct of aspartyl 61 in a small, chloroform-methanol soluble protein of F_O 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 phage P1 *cm1100* as described by Miller (1972). Most of the strains used are isogenic Ilv^+/*unc* cotransductants of strain AN346 (F^-, *entA403, pyrE41, ilvC7, argH1, rpsL109, supE44*; Gibson *et al.*, 1977). These transductants include strain MM180 [unc^+], strain MM128 [*uncE101* (A24S)] and strains carrying the *uncE102* (A24S), *uncE103* (A24S) and *uncE104* (A24S) alleles (Fillingame, 1979). Some strains were made *recA56* by phage P1 transduction as described by Mosher *et al.* (1983). The *recA56* strains include: MM833 [unc^+], MM441 [*uncE107* (D61N)], and MM994 [*uncE114* (Q42E)]. Strain MM349 [*uncB402*] is a derivative of strain RR1 [F^-, *pro, leu, thi, lacY, rpsL, hsd20* (r_B^-, m_B^-); Bolivar and Backman, 1979] that was made *recA56*

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

by Dr. Mary Mosher in this laboratory. Strains DC13 (I28T) and DC25 (I28V), described in Hoppe *et al.* (1980), are Ilv$^+$, Met$^+$ derivatives of *E. coli* strain Ymel (λ) [F$^-$, *lacI, fadR, but12, rha*] and were a gift of Dr. John E. G. McCarthy (Gesellschaft für Biotechnologische, Braunschweig, Federal Republic of Germany).

Growth of Cells—Minimal medium 63, LB medium, and 2YT medium are described in Miller (1972). M63 minimal medium agar plates were supplemented with 2 μg/ml thiamine hydrocholoride, 0.2 mM uracil, 0.2 mM L-arginine, and 40 μM 2,3 dihydrobenzoic acid as required, and 0.2% D-glucose was used as a carbon source. A combination of 0.6% disodium succinate hexahydrate, 0.2% potassium acetate, and 0.2% potassium L-malate 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-malate 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 minimal medium supplemented with succinate-acetate-malate as carbon sources and any of the required supplements as indicated above. Plasmid transformants were selected, and the plasmids were maintained by the addition of ampicillin to media at 100 μg/ml.

Molecular Genetic Methods—Plasmid pBR322 and phages M113mp18 and M13mp19 are described in Maniatis *et al.* (1982). Chromosomal DNA was isolated by the method of Silhavy *et al.* (1984), and the 4.3-kilobase *Hin*dIII fragment of *unc*-derived DNA was cloned into plasmid pBR322. The *Bam*HI to *Hpa*I fragment of *uncE* DNA was cloned between the *Bam*HI and *Hin*cII sites of phages M13mp19 and M13mp19, and the DNA sequence of the *uncE* gene was determined as described previously by Mosher *et al.* (1985).

Biochemical Methods—Membranes were prepared with a French press in TMDG buffer (50 mM Tris-HCl, pH 7.5, 5 mM $MgCl_2$, 1 mM dithiothreitol, 10% (w/v) glycerol) supplemented with 6 mM *p*-aminobenzamidine HCl (Hermolin *et al.*, 1983). The methods of Hermolin and Fillingame (1989) were followed for purification of subunit *c* in chloroform-methanol and for anion exchange HPLC analysis of charge variants of subunit *c*.

Inhibitor Treatment and ATPase Assays—The method used for DCCD treatment is that described by Fillingame (1979). Membranes (5–12 μg of protein) were incubated in 0.9 ml of Tris-Mg^{2+} assay buffer (55.5 mM Tris H_2SO_4, pH 7.8, 0.222 mM $MgSO_4$) 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 [^{32}P]ATP. After a 6-min incubation the reaction was terminated by the addition of trichloroacetic acid. The molybydate complex of [γ-^{32}P]phosphate was extracted into isobutyl alcohol-benzene (Fillingame, 1975). Under these assay conditions, 20–30% of the membrane APTase proves to be DCCD insensitive because of dissociation of F_1 from F_O (Hermolin and Fillingame, 1989; Fraga and Fillingame, 1989).

To test venturicidin inhibition, membranes (125 μg of protein in 25 μl of TMDG buffer) were incubated in 0.95 ml of 10 mM Hepes-KOH, pH 7.5, 5 mM $MgCl_2$, 300 mM KCl and treated with 10 μl of venturicidin in dimethyl sulfoxide, or dimethyl sulfoxide only, for 10 min at 25 °C. The ATPase assay was then initiated by addition of 25 μl of 40 mM [γ-^{32}P]ATP. After 5 min, the assay was terminated by the addition of trichloroacetic acid. [^{32}P]Phosphate was extracted as described above. The Hepes-Mg^{2+}-KCl assay buffer used here is that described by Paule and Fillingame (1989). Less than 10% of membrane-bound F_1 dissociates from F_O under these incubation conditions.

RESULTS

Localization of Mutations Conferring DCCD Resistance—Chromosomal DNA was isolated from four independently selected DCCD-resistant mutants and the DNA digested with *Hin*dIII. The *Hin*dIII fragment of chromosomal DNA containing the *uncBEFHA* genes (bases 870–5308)[2] was cloned into the *Hin*dIII site of plasmid pBR322. Recombinant plasmid transformants containing the wild-type *uncB* gene were selected based upon complementation of the *uncB402* chromosomal recipient strain MM349.

To determine whether a mutation in the *uncE* gene was responsible for DCCD resistance, the *Bam*HI(1727) to *Hpa*I(2162) DNA fragment containing *uncE* was cloned from the plasmids described above into the *Bam*HI and *Hin*cII sites of phage M13mp18 and the *uncE* DNA sequenced. 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 Ala^{24} → Ser substitution was the mutation causing DCCD resistance, the *Bam*HI(1727) to *Hpa*I(2162) fragment of *uncE101*(A24S) DNA was subcloned from the larger plasmid described above into the *Bm*HI and *Pvu*II sites of plasmid pBR322. This plasmid (pMO101) only weakly complemented *uncE* recipient strains and did not confer DCCD-resistant growth. In contrast, plasmid pCP35 (Fillingame *et al.*, 1984), which was thought to be equivalent to plasmid pMO101 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 *Bam*HI to *Hpa*I DNA fragment was cloned into the *Bam*HI and *Pvu*II sites of plasmid pBR322, and, in contrast to plasmid pCP35, this plasmid (pMO100) 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 pMO101 plasmids were exchanged. The plasmids were cut with *Bam*HI and at the *Nde*I site (position 2296) in the pBR322 vector DNA (Fig. 1). The 655-base pair *Bam*HI/*Nde*I fragment containing the *uncE* gene was separated from the 2449-base pair *Bam*HI/*Nde*I vector fragment by agarose gel electrophoresis, and the purified fragments were mixed and ligated. The product plasmid pMO351 contains the *uncE101* (A24S) gene in the pCP35 *Bam*HI/*Nde*I vector DNA. Plasmid pMO351 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 pMO101-derived vector DNA complemented poorly and confirmed that the differences in complementation efficacy were caused by the vector DNA.

The *Eco*RI(4361) to *Bam*HI(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 $(T)_{132}$GGATGC$(T)_{139}$. It is unclear from the DNA sequence whether T_{132} or T_{139} was deleted. The

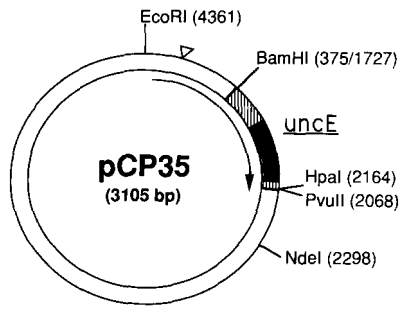

FIG. 1. **Plasmic pCP35 and related plasmids.** Plasmid pCP35 was constructed by ligating the 435-base pair *Bam*HI/*Hpa*I fragment of wild-type *uncE* DNA between the *Bam*HI and *Pvu*II sites of plasmid pBR322. The *black area* shows the *uncE* structural gene, and the *hatched area* is other *unc* DNA. The isolated pCP35 vector DNA was found to contain a 7-base pair deletion between bases 132 and 139 clockwise of the *Eco*RI site (Δ). The initiation site and direction of transcription from the tetracycline resistance promoter are shown. Plasmids pMO100 and pMO101 contain the *Bam*HI/*Hpa*I fragment of *uncE*$^+$ or *uncE101* DNA in a normal pBR322 vector. Plasmid pMO351 was constructed by ligating the *Bam*HI → *Nde*I fragment of *uncE101* DNA from plasmid pMO101 into the *Bam*HI and *Nde*I sites of vector DNA from plasmid pCP35. *bp*, base pairs.

[2] The nucleotide numbering used for the *unc* operon is that given by Walker *et al.* (1984).

TABLE I

Conferral of DCCD resistance by uncE101 (A24S) gene is dependent upon plasmid vector

Plasmid	*uncE* allele[a]	Vector[b]	Growth of transformants on succinate[c]					
			Wild type		*uncE114*		*uncE107*	
			−DCCD	+DCCD	−DCCD	+DCCD	−DCCD	+DCCD
pMO100	W.T.	pBR322	+++	−	+	−	±	−
pMO101	*E101*	pBR322	+++	−	+	−	±	−
pCP35	W.T.	pCP35	+++	−	+++	−	+++	−
pMO351	*E101*	pCP35	+++	++	+++	++	+++	++

[a] *Bam*H(1727) → *Hpa*I(2162) fragment of *unc* DNA. W.T., wild type; *E101*, A24S.
[b] *unc* DNA cloned between *Bam*HI(375) and *Pvu*II(2065) sites of plasmid pBR332.
[c] Scoring (+++, ++, +) indicates the extent of growth on succinate as carbon source; (±) indicates very weak to negligible growth; (−) indicates no growth.

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[a]	Colony size[b]	Yield of subunit c			Ratio of subnit c product (plasmid/ chromosomal)
		Total	Q42E (chromosomal)	A24S (plasmid)	
		μg/mg cellular protein			
pMO351/MM994	100% large	2.07	0.42	1.65	4.0
pMO101/MM994	50% large 50% small	1.05	0.40	0.65	1.6
pMO101/MM994	>95% small	0.54	0.34	0.20	0.6

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

deletion lies in the tetracycline resistance gene and conceivably could affect expression of the cloned *uncE* gene from the tetracycline resistance promoter (Stuber and Bujard, 1981).

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*, A24S) or plasmid pMO351 (*uncE101*, A24S) 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 pMO1010 transformants were distinctly smaller.[3] 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 1,200 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

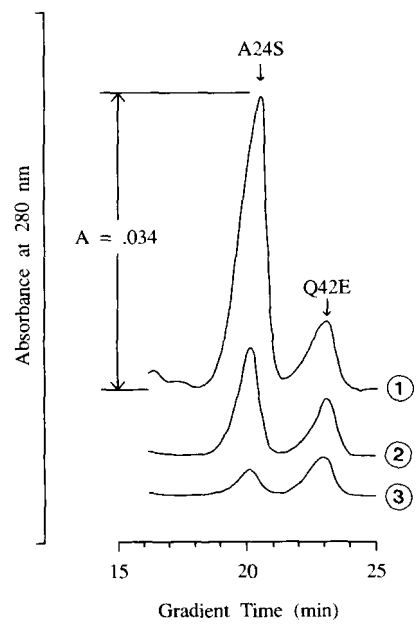

FIG. 2. **Separation of A24S, plasmid-derived subunit *c* from Q42E, chromosomal-derived subunit *c* by HPLC anion exchange chromatography.** The purified subunit *c* fraction from A24S-producing plasmid transformants of strain MM994 (chromosomal Q42E) were separated on an AX300 anion exchange HPLC column 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).

[3] The pMO101 and pMO351 plasmid transformants of wild-type strain MM833 (*unc*$^+$) did not differ in size.

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.[4]

Mixing of A24S and Q42E Subunits in F_O Restores DCCD Sensitivity to ATPase—The *uncE101* (A24S) mutant shows DCCD-resistant ATPase activity because of reduced reactivity of DCCD with Asp^{61} (Fillingame, 1975). The *uncE114* (Q42E) mutant exhibits DCCD-resistant ATPase activity because of uncoupling of F_1 and F_O (Mosher *et al.*, 1985). Membranes from *uncE101* 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.[4]

Comparison of DCCD Resistance Conferred by the A24S, I28T, and I28V Mutations—Hoppe *et al.* (1980) had previously reported DCCD-resistant mutants with substitutions at Ile^{28} 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).

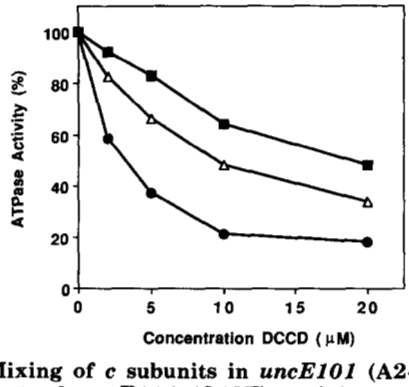

FIG. 3. **Mixing of *c* subunits in *uncE101* (A24S) plasmid transformant of *uncE114* (Q42E) recipient cell leads to DCCD-sensitive hybrid ATPase.** Membranes were incubated for 20 min at 30 °C with DCCD in Tris-Mg^{2+} assay buffer, and ATPase activity was assayed as described under "Experimental Procedures." The results shown are the average of two experiments. ●, wild-type membrane (strain MM180); ■, *uncE101* (A24S) membrane (strain MM128); △, membrane from plasmid pMO351 (A24S) transformant of strain MM994 (Q42E) (strain MO91).

[4] When strain pMO351/MM994 was grown on a glucose minimal medium containing 5% LB, the ratio of plasmid to chromosomal product increased to 8–9, and the total yield of subunit *c* increased to approximately 5 μg/mg cellular protein. Despite the large overproduction of subunit *c*, only tiny amounts were found in the cytoplasmic fraction of transformant cells (<3% of that found in the membrane), *i.e.* the amount found in the cytoplasm did not exceed that of normal monoploid cells.

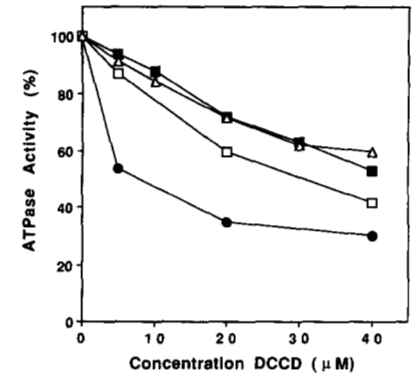

FIG. 4. **Comparison of the DCCD resistance conferred by the A24S, I28T, and I28V mutations.** Membranes were treated with DCCD and assayed for ATPase activity as described in Fig. 3. The results shown are the average of three experiments. ●, wild-type membrane (strain MM180); ■, A24S membrane (strain MM128); □, I28V membrane (strain DC13); △, I28T membrane (strain DC25). The specific ATPase activities of the membranes were: 0.45 (wild type), 0.86 (A24S), 0.85 (I28V), 0.76 (I28T).

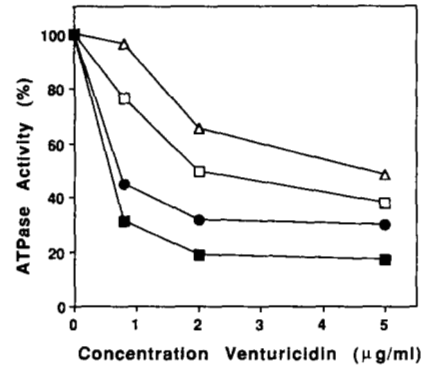

FIG. 5. **Contrasting effects of Ile^{28} and Ala^{24} mutations on venturicidin sensitivity of membrane F_1F_O ATPase activity.** Membranes were treated with venturicidin in Hepes-Mg^{2+}-KCl assay buffer and assayed for ATPase activity as described under "Experimental Procedures." ●, wild-type membrane (strain MM180); ■, A24S membrane (strain MM128); □, I28V membrane (strain DC13); △, I28T membrane (strain DC25).

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 I28V or I28T 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'*-nitronitrosoguanidine. The mutants isolated by Friedl *et al.*

(1977) were selected after *N*-methyl-*N'*-nitronitroguanidine mutagenesis on plates containing 24 mM DCCD and a succinate carbon source. The codon changes predicted for the I28V and I28T mutations are ATC to GTC and ACC, respectively. We have confirmed these substitutions by DNA sequence analysis of chromosomal DNA amplified by the polymerase chain reaction. These mutants were therefore generated by AT → GC transition mutations. Each of the A24S substitutions was caused by a GCT to TCT codon change, *i.e.* by GC → TA transversion mutations. It is of interest that the major and direct mode of MNNG mutagenesis is via GC → AT transitions (Glass, 1982) and that none of the mutants contain this type of substitition. GC → AT transitions would lead to silent changes in the Ile^{28} codon but would cause threonine or valine substitutions in the Ala^{24} codon. One might predict that threonine or valine substitutions at position 24 will either have a deleterious effect on ATP synthase function or not confer DCCD resistance.

The finding that mutations in both Ala^{24} and Ile^{28} affect reactivity of DCCD with Asp^{61} 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 Asp^{61} 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 $iet 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 Asp^{61} during the proton transfer cycle (Miller *et al.*, 1990; Fillingame, 1990). The Gly^{23} and Gly^{27} residues of transmembrane helix-1 are absolutely conserved, and it is of interest that these glycines immediately precede the two positions giving rise to DCCD resistance. In addition, glycine is usually found at positions 25 and 29. The high frequency of conserved glycine suggests that a compact structure may be necessary. The region is also very hydrophobic. The introduction of a serine or threonine residue could obviously disrupt DCCD binding by decreasing the hydrophobicity of the binding pocket. The I28V mutation is more difficult to rationalize but may again indicate the necessity for a precisely packed, compact structure in this region.

Ala^{24} and Ile^{28} are clearly important for DCCD binding in *E. coli* subunit *c* but are unlikely to be the only determinants of binding specificity. Several species of subunit *c* have serine at the equivalent of position 24, and others have valine or threonine at the equivalent of position 28 (Fig. 6). These changes by themselves do not lead to DCCD resistance, *e.g.* both bovine mitochondria with the equivalent of Ser^{24} and *Rhodospirillum rubrum* with the equivalents of Ser^{24} and Val^{28} are DCCD sensitive (Beechey *et al.*, 1967; Bengis-Garber and Gromet-Elhanan, 1979). In addition, the binding site must have components that discriminate between structurally different carbodiimides. This was indicated by the studies of Abrams and Baron (1970) in which a specificity for nonpolar carbodiimides was first shown. However, the inhibitory potence of various carbodiimides does not appear to be a simple function of their hydrophobicity. The relative inhibitory potence of apolar carbodiimides does vary widely (Patel and Kaback, 1976; Beechey and Knight, 1978; Hoppe and Sebald, 1984) and seems to differ somewhat depending upon the species of F_1F_O examined. These observations are most easily explained by a very specific or rigidly shaped binding site within subunit *c*.

Interpretation of the carbodiimide inhibition experiments is complicated by the complexity of the reaction. DCCD must first react with the carboxylate form of Asp^{61} to yield on *O*-acylisourea and the adduct then rearrange to form the stable *N*-acylurea product (Williams and Ibrahim, 1981):

$$R\text{-}CO_2^- + H^+ + DCCD \rightarrow O\text{-acylisourea} \quad (1)$$

$$O\text{-acylisourea} \rightarrow N\text{-acylurea} \quad (2)$$

Reaction 1 might only occur after DCCD was bound at its binding site. Alternatively, reaction 1 might occur between an Asp^{61} 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*-acylisourea to dicyclohexylurea that would regenerate a free carboxyl group.

Perlin *et al.* (1985) have shown that venturicidin inhibits the activity of *E. coli* F_1F_O-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_1F_O-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 I28T 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

	Amino Acid Number									
	20	21	22	23	24	25	26	27	28	29
E. coli	**Ala**	**Ala**	**Ile**	**Gly**	**Ala**	**Ala**	**Ile**	**Gly**	**Ile**	**Gly**
S. faecalis	**Ala**	**Ala**	**Ile**	**Gly**	**Ala**	Gly	Tyr	**Gly**	Asn	**Gly**
M. laminosus	**Ala**	**Ala**	**Ile**	**Gly**	Pro	Gly	**Ile**	**Gly**	Gln	**Gly**
A. caldarius	**Ala**	**Ala**	Val	**Gly**	Ser	Gly	Val	**Gly**	Asp	**Gly**
PS-3	Gly	**Ala**	Leu	**Gly**	**Ala**	Gly	**Ile**	**Gly**	Asn	**Gly**
R. rubrum	Gly	Met	**Ile**	**Gly**	Ser	Gly	**Ile**	**Gly**	Val	**Gly**
Synechococus	**Ala**	**Ala**	**Ile**	**Gly**	Pro	Gly	**Ile**	**Gly**	Gln	**Gly**
Spinach chlorop.	**Ala**	Ser	**Ile**	**Gly**	Pro	Gly	Val	**Gly**	Gln	**Gly**
Tobacco mito.	**Ala**	Ser	Ala	**Gly**	**Ala**	**Ala**	**Ile**	**Gly**	**Ile**	**Gly**
Maize mito.	**Ala**	Leu	Ala	**Gly**	**Ala**	**Ala**	Val	**Gly**	**Ile**	**Gly**
Yeast mito.	Gly	Leu	Leu	**Gly**	**Ala**	Gly	**Ile**	**Gly**	**Ile**	Ala
N. crassa mito.	Gly	Leu	Thr	**Gly**	**Ala**	Gly	**Ile**	**Gly**	**Ile**	**Gly**
A. nidulans mito.	Gly	Leu	Gly	**Gly**	**Ala**	Gly	**Ile**	**Gly**	Thr	**Gly**
Bovine mito.	Gly	Val	Ala	**Gly**	Ser	Gly	Ala	**Gly**	**Ile**	**Gly**
Consensus	Ala/Gly	X	X	**Gly**	X	Gly	X	**Gly**	X	Gly

FIG. 6. **Sequence comparisons for the conserved glycine-rich segment of transmembrane helix-1 of subunit c from different species.** The amino acid numbering system is for the *E. coli* protein. Residues that are identical to those in *E. coli* are printed in *boldface*. The sequences are from the following sources. *E. coli*, *Streptococcus faecalis*, *Mastigocladus laminosus*, *Acido caldarius*, thermophilic bacterium PS-3, *R. rubrum*, spinach chloroplast, *Saccharomyces cerevisiae* mitochondria, *Neurospora crassa* mitochondria, *Aspergillus nidulans* mitochondria, and bovine mitochondria: Hoppe and Sebald, 1984; *Synechococus* 6301: Cozens and Walker, 1987; tobacco mitochondria and *Zea mays* mitochondria: Bland *et al.*, 1986.

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_1F_O-ATPase.

Our unpublished NMR studies on isolated subunit *c* in chloroform-methanol solvent indicate an extended α-helix from Asp^7 through Leu^{31}.[5] 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 Asp^{61} with DCCD even when the protein is purified. The conserved Gly^{23} and Gly^{27} residues would lie on one face of this α-helical structure and $Gly(Ala)^{25}$ and $Gly(Ala)^{29}$ 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, 1990), one could easily envision a rotation of helices relative to each other. For example, in one state the Val^{60} and Met^{57} side chains of one subunit *c* could lie in the groove formed by Gly^{23} and Gly^{27} 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 Ala^{25} and Gly^{29}.

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_1 (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_1. Mutations in Asp^{61} 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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