STRUCTURAL GENE CODING FOR THE DICYCLOHEXYLCARBODIIMIDE-BINDING PROTEIN OF THE PROTON-TRANSLOCATING ATPase FROM *ESCHERICHIA COLI*

Locus of the gene in the F_1–F_0 gene cluster

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1. Introduction

The proton-translocating ATPase (F_1–F_0) is an apparatus for the synthesis of ATP common to different energy-transducing organelles, such as mitochondria, chloroplasts and bacterial cytoplasmic membranes (reviews [1–4]). The ATPase consists of two portions, F_1 and F_0. F_1 is a peripheral membrane component with 5 different subunits ($\alpha,\beta,\gamma,\delta,\epsilon$), and it is bound to the integral membrane component, F_0, which functions as a proton pathway. Two or more subunits have been found in F_0 from different sources. The proteolipid subunits, dicyclohexylcarbodiimide (DCCD)-binding proteins, have been purified from several organisms [2,5,6] and their primary sequences have been determined [7].

Two types of *E. coli* mutant of this subunit, which both result in inability to bind DCCD, have been reported; the F_0 of mutant strain DG7/1 is defective in proton translocation [8,9], whereas that of the other type of mutant RF7 [10] is normal. Thus DG7/1 cannot grow with succinate as the sole carbon source because of defective oxidative phosphorylation, whereas RF7 can grown normally. Both mutant strains have functional F_1, although their membrane ATPase activities are not sensitive to DCCD because the mutant protein cannot bind this compound. DCCD binds to an aspartyl residue at position 61 of the polypeptide, and this residue is replaced by a glycine residue in the protein of DG7/1 [8,9]. It has not been shown directly whether these mutations are in the gene cluster coding for components of F_1–F_0 [11], because the structural gene for DCCD-binding protein has not yet been mapped in detail. It is difficult to map this component because F_1–F_0-containing both mutant and wild-type proteins showed impaired F_0 function [12]. Transducing phage λ*asn*-5 carries a whole set of structural genes for F_1–F_0 [12], and these genes were localized on a defined portion ($\sim 4.5 \times 10^6$ M_r) of *E. coli* DNA using various plasmids and transducing phages [13]. The structural gene for the DCCD-binding protein has not yet been located on the DNA segment.

Here, we have studied the genetic complementation of the two mutants with transducing phages and hybrid plasmids carrying 3 different portions of the gene cluster coding for F_1–F_0. In detailed biochemical analysis of membranes, we found that a hybrid plasmid pMCR533 could complement both mutants. From analysis of DNA of the plasmid, we concluded that the structural gene for the DCCD-binding protein is located within a DNA fragment (1.38×10^6 M_r) extending from the promoter proximal *Hin*dIII site to the *Eco*RI site.

Abbreviations: F_1–F_0, proton-translocating ATPase; DCCD, dicyclohexylcarbodiimide

2. Materials and methods

The *E. coli* strains used were all derivates of K12: DG7/1, *lacI fadR*, *but12*, *rha*, *ilv*, *met* [8]; RF7, *argE3*, *thi*-1, *strR*, *dcc*-1 [10]; OK1008, *recA* derivative of RF7 constructed in this study; AN180, *argE3*, *thi*-1, *strR* [14]; W3110, wild-type. Cells were grown in L broth [15] or a minimal medium [13] supplemented with carbon sources as in the figure legends

or in the text. Growth of cells was followed by monitoring turbidity at 650 nm. The defective transducing phage λ*asn*-5 and the hybrid plasmid pMCR533 carrying genes for F_1–F_0 were obtained as in [12].

The hybrid plasmid pAT1 and pFT302 were constructed by inserting a fragment of λ*asn*-5 DNA digested with restriction endonucleases *Hin*dIII and *Eco*RI into the high copy number plasmids pBR322 [16] and pMCR566 (a chloramphenicol-resistant derivative of pACYC177 [17], constructed by T. Miki), respectively. Transformation of mutants with plasmids was carried out as in [13].

Membrane vesicles were prepared by disrupting cells in a French press as in [12]. ATP-dependent H^+ translocation was assayed by measuring quenching of quinacrine fluorescence when ATP was added to vesicles [12]. Other procedures including preparation and analysis of DNA and measurement of ATPase activity were as in [12,13]. Restriction endonucleases *Eco*RI and *Hin*dIII were from Boehringer-Mannheim. The reagents used were the highest grade available commercially.

3. Results and discussion

3.1. *Complementation of DG7/1 with pMCR533*

We tested several transducing phage [13] including λ*asn*-5 for complementation of DG7/1. These phages were shown to carry various portions of the gene cluster coding for F_1–F_0, but none of the transductants of the mutant by these phages were able to grow on succinate (not shown), suggesting that these phages could not complement the mutant. The structural genes for F_1–F_0 in the lysogen were supposed to be diploid with the wild-type allele (on the prophage) and mutant allele (on the DG7/1 chromosome). Thus our observation is consistent with [18], where a diploid strain carrying the wild-type allele of the DCCD-binding protein on the chromosome and the mutant allele of F′ plasmid was shown to have the mutant phenotype. These results suggest that the protein functions as an oligomer and that the hybrid oligomer consisting of wild-type and mutant proteins (presumably in a 1:1 ratio) is defective in DCCD binding.

We tested plasmids carrying various portions of the gene cluster for complementation. The portions of the gene cluster carried by pAT1, pMCR533 and pFT302 are summarized in fig.1. The promoter for

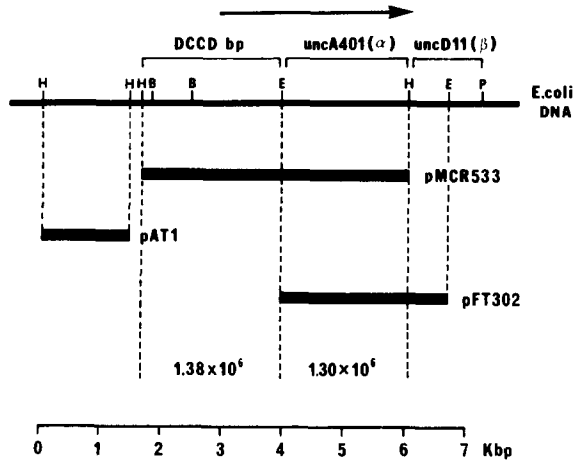

Fig.1. Structural gene locus for DCCD binding protein on the physical map of *E. coli* DNA. The physical map of the bacterial DNA around the gene cluster coding for F_1–F_0 is shown at the top of the figure. The approximate positions of the mutant alleles *uncA401* [14] and *uncD11* [20] determined in [13] are shown. The structural gene locus of DCCD-binding protein (DCCD bp) determined in this study is also shown. The *uncB402* mutation [22] is within the same DNA segment as the gene locus of the DCCD-binding protein. However, it was not possible to distinguish these 2 genes here. This portion of DNA is located around 82.5 min on the chromosome linkage map [21]. The abbreviations used for endonuclease cleavage sites are: (H) *Hin*dIII; (B) *Bam*HI; (E) *Eco*RI; (P) *Pst*I. A more detailed physical map of this region has been reported [13]. The portions of the *E. coli* DNA carried by plasmids pAT1, pMCR533 and pFT302 are indicated by solid lines. The arrow indicates the direction of transcription.

the gene cluster is located on the right side of pAT1 or left side of pMCR533 (fig.1) [13]. Ampicillin-resistant transformants of the mutant by these plasmids were selected and >10 independent colonies were tested for growth in minimal medium with succinate. This medium supported growth of all the transformants of pMCR533, but not of any of the transformants of pAT1 or pFT302. The typical results shown in fig.2 suggest that the gene for the DCCD-binding protein is on pMCR533 and that the effect was complemented. This notion was confirmed by biochemical analysis of membranes; formation of a proton-motive force dependent on ATP was analyzed in membrane vesicles of the transformants (table 1). As expected, only pMCR533 transformants had the wild-type level of activity of proton translocation, indicating that pMCR533 has the gene for the DCCD-binding protein. The plasmid (pMCR533) studied here

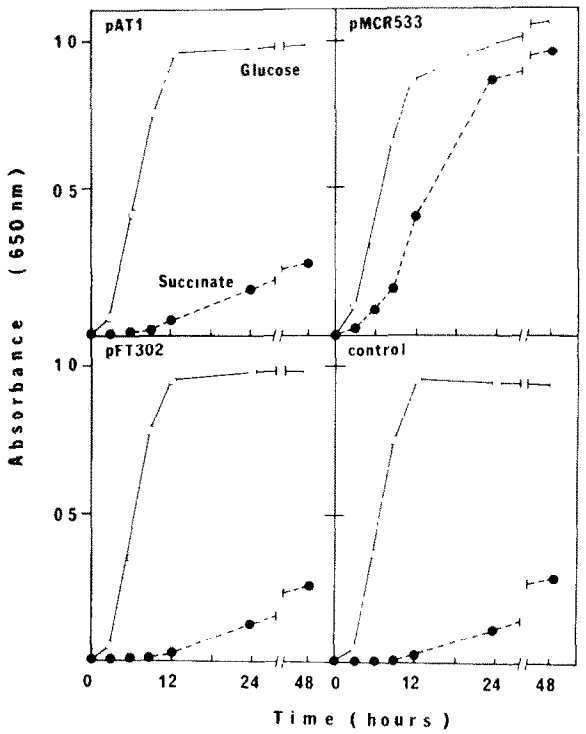

Fig.2. Growth of various transformants of DG7/1 with succinate as the sole carbon source. The mutant (DG7/1) was transformed by plasmids carrying 3 different portions of the gene cluster coding for F_1–F_0 as shown in fig.1. Transformants with each plasmid were selected by ampicillin resistance and purified. Typical examples of growth of these transformants are shown. Overnight cultures of DG7/1 transformed by pAT1, pMCR553 grown in L broth were inoculated into minimal medium containing 0.4% succinate (•) as the sole carbon source (0.1% inoculum) and incubated at 37°C. Growth was followed by measuring the turbidity of the culture at 650 nm. Growth of DG7/1 was also studied by the same procedure (bottom right). As a control each strain was cultured in the same salt medium supplemented with 0.2% glucose (○).

had a higher copy number than the F′ plasmid in [18] (not shown). The reason why we observed complementation with pMCR533 was probably that the number of normal molecules was higher than that of the defective ones in the transformant membrane (see section 3.2).

3.2. *Complementation of RF7 with pMCR533*

To confirm the above results, we examined complementation with another mutant (RF7). The growth of RF7 was completely insensitive to 25 mM DCCD, confirming [10]. The pFT302 and pAT1

Table 1
Formation of protonmotive force dependent on ATP in membrane vesicles from various transformants of DG7/1

Bacterial strain	Plasmid	Fluorescence quenching ($\Delta F \cdot min^{-1} \cdot mg\ protein^{-1}$)
1. DG7/1	None	1.1
	pAT1	4.4
	pFT302	3.4
	pMCR533	51.2
2. W3110	None	55.1
3. RF7	None	66.0

Formation of a protonmotive force was estimated by quenching of quinacrine fluorescence (ATP-dependent) using membrane vesicles prepared from various transformants of DG7/1. Each assay mixture contained 0.20 mg vesicle protein and results are expressed as % change of fluorescence intensity/mg membrane protein ($\Delta F \cdot min^{-1} \cdot mg\ protein^{-1}$). As controls, vesicles from strains DG7/1, W3110 (wild-type) and RF7 (DCCD-binding protein mutant, but having an active F_0) were assayed under the same conditions. See text for details

transformants had essentially the same insensitivities to DCCD. However, growth of the pMCR533 transformant was sensitive to DCCD; the turbidities of cultures of RF7, AN180 and RF7/pMCR533 in the presence of 25 mM DCCD were 0.4, 0.15 and 0.15, respectively, when overnight cultures of these strains were incubated for 6 h in a fresh medium supplemented with succinate (initial absorbance of 0.02). The membrane ATPase of pMCR533 transformant was sensitive to DCCD, whereas that of the pAT1 and pFT302 transformants was not (fig.3). The ATPase activity of the pMCR533 transformant is significantly less sensitive to DCCD than that of the wild-type, possibly because the mutant proteins are in the transformant membrane, although most of the proteins are from the wild-type gene. In this connection it would have been of interest to reconstitute liposomes with various ratios of wild-type and mutant DCCD-binding proteins and to study the function of their F_0. However, other components seemed to be required to reconstitute F_0 in vitro [2] and reconstitution from individual components was not successful. Proton translocation (ATP-dependent) in membrane vesicles of the pMCR533 transformant was also sensitive to DCCD (not shown). It must be noted that essentially the same results were obtained using the mutant allele RF7 in a *recA*$^-$ or *recA*$^+$ background. Thus pAT1 or pFT302 could not convert RF7 to DCCD-sensitive phenotype, possibly the whole cistron of DCCD-

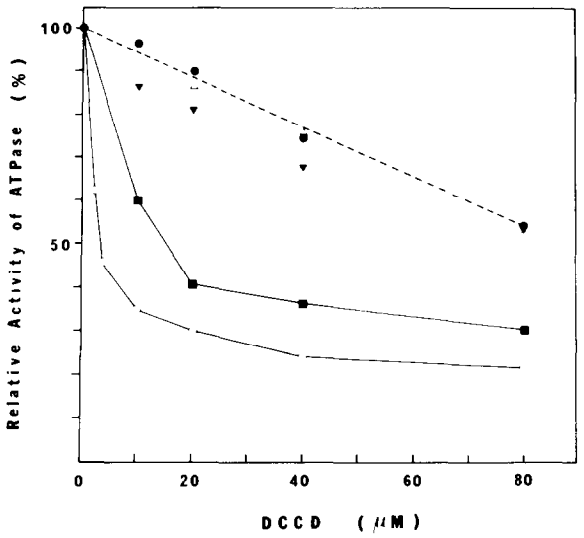

Fig.3. Effect of DCCD on membrane ATPase from RF7 and its transformants. The *recA* derivative of RF7 (strain OK1008) was transformed by plasmids carrying the 3 different portions of the gene cluster coding for the F_1–F_0 (fig.1). Transformants with each plasmid were selected and purified as in fig.2. Membranes were prepared from each transformant as in [12] and ATPase activity was measured in the presence of various concentrations of DCCD. Results are relative activities of membranes from transformants by pMCR533 (■), pAT1 (△), pFT302 (▼) and control membranes from RF7 (●) and AN180 (○). Control membrane ATPase activities (without DCCD) were: pMCR533 transformant, 1.25; pAT1 transformant, 1.28; pFT302 transformant, 1.28; RF7, 1.13; AN180, 1.25 expressed as units/mg protein. One unit of ATPase was defined as the amount hydrolyzing 1 μmol ATP/min [12].

binding protein being on pMCR533 DNA. Absence of the gene coding for DCCD-binding protein in pAT1 and pFT302 was confirmed (see section 3.3).

3.3. *Conclusion*

Results using two mutants indicated that pMCR533 carries the whole cistron of the DCCD-binding protein. Comparison of the restriction map of this plasmid with those of two other plasmids (fig.1) suggests that the gene coding for this protein is located in a DNA fragment of 1.38×10^6 M_r extending from the promoter proximal *Hin*dIII site to the *Eco*RI site as shown in fig.1. To our knowledge, this is the first indication that the structural gene for the DCCD-binding protein is located in a defined portion of the gene cluster of F_1–F_0, although λ*asn*-5 has been suggested to carry this gene because its lysogen could overproduce a protein with the same M_r as the DCCD-binding protein [19]. At present it is difficult to distinguish this gene from *uncB* (a gene coding for an unidentified F_0 component) [22], but it is located closer to the putative promoter site than the *uncA* gene [14]. In preliminary sequencing studies we found that the nucleotide sequence of the carboxyl terminal of the DCCD-binding protein is in the middle of the 1.38×10^6 M_r fragment (fig.1), confirming the results of this study. Although pMCR533 complemented RF7 (*recA*), it was unable to transform *uncA401*(*recA*) as discussed in [13]. The reason for this discrepancy is unknown. Studies on the regulation of this gene cluster are in progress.

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