Random mutagenesis of the gene for the β -subunit of F_1 -ATPase from *Escherichia coli*

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ATP synthesis by oxidative phosphorylation in *Escherichia coli* occurs in catalytic sites on the β -subunits of F_1 -ATPase. Random mutagenesis of the β -subunit combined with phenotypic screening is potentially important for studies of the catalytic mechanism. However, when applied to haploid strains, this approach is hampered by a preponderance of mutants in which assembly of F_1 -ATPase *in vivo* is defective, precluding enzyme purification. Here we mutagenized plasmids carrying the *uncD* (β -subunit) gene with hydroxylamine or *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine and isolated, by phenotypic screening and complementation tests, six plasmids carrying mutant *uncD* alleles. When the mutant plasmids were used to transform a suitable *uncD*[−] strain, assembly of F_1 -ATPase *in vivo* occurred in each case. Moreover, in one case (β Gly-223 → Asp) F_1 -ATPase assembly proceeded although it had previously been reported that this mutation, when present on the chromosome of a haploid strain, prevented assembly of the enzyme *in vivo*. Therefore, this work demonstrates an improved approach for random mutagenesis of the F_1 - β -subunit. Six new mutant *uncD* alleles were identified: β Cys-137 → Tyr; β Gly-142 → Asp; β Gly-146 → Ser; β Gly-207 → Asp; β Gly-223 → Asp; and a double mutant β Pro-403 → Ser, Gly-415 → Asp which we could not separate. The first five of these lie within or very close to the predicted catalytic nucleotide-binding domain of the β -subunit. The double mutant lies outside this domain; we speculate that the region around residues β 403–415 is part of an α - β intersubunit contact surface. Membrane ATPase and ATP-driven proton pumping activities were impaired by all six mutations. Purified F_1 -ATPase was obtained from each mutant and shown to have impaired specific ATPase activity.

INTRODUCTION

The enzyme F_1 -ATPase is responsible for ATP synthesis by oxidative phosphorylation in bacteria and mitochondria and by photophosphorylation in chloroplasts. The subunit structure of F_1 -ATPase is $\alpha_3\beta_3\gamma\delta\epsilon$, and the catalytic sites are contained on the β -subunits (reviewed in Senior, 1988). Mutational analysis represents an important approach to understanding the catalytic mechanism of ATP synthesis because it provides a procedure for obtaining homogeneous chemically-modified variants of this large and complex enzyme.

In *Escherichia coli* the *uncD* gene encodes the F_1 - β -subunit (Fayle *et al.*, 1978). Unfortunately, only a small fraction (~20%) of chromosomal *uncD* mutants were able to assemble F_1 in haploid *E. coli* cells (Senior *et al.*, 1979, 1983; Kanazawa *et al.*, 1983; Noumi *et al.*, 1986). Site-directed mutagenesis has provided one means of circumventing this difficulty and obtaining additional mutations in *uncD* which yield properly-assembled, catalytically-impaired enzyme variants (e.g. Parsonage *et al.*, 1987b). In order to obtain more β -subunit mutants we have also tried the technique of random chemical mutagenesis of plasmid-encoded *uncD* gene, using *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine and hydroxylamine as mutagens. The results of these studies, with characteristics of new mutants obtained, are described in this paper.

MATERIALS AND METHODS

Plasmids and *E. coli* strains

The plasmids used are listed in Table 1. The *E. coli* strains and genotypes are listed in Table 2.

Mutagenesis with *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine

Strain AN869 was transformed with either plasmid pAN45 or pAN36. The resultant strains were grown in 200 ml of minimal medium/glucose/chloramphenicol to mid-log phase, spun down, washed once in 0.1 M-sodium citrate buffer, pH 6.0, and resuspended in 20 ml of the same buffer. Solid mutagen was added to give a final concentration of 100 μ g/ml. The cells were incubated at 37 °C for 20 min with slow shaking and were then washed twice in minimal medium, resuspended in 30 ml of Luria broth (LB)/glucose/chloramphenicol medium and incubated for 2.5 h at 37 °C. Cells were washed once in minimal medium, then resuspended in 1 litre of minimal medium/casamino acids/glucose/chloramphenicol and shaken vigorously at 37 °C for 4.5 h. Plasmid preparations were made from the resultant cell suspensions.

Mutagenesis with hydroxylamine

Plasmid pRPG32 (200 μ g/ml) was incubated in 500 μ l of 0.8 M-hydroxylamine/0.1 M-sodium phosphate (pH 6.0)/1 mM-EDTA for 2 h at 65 °C. The plasmid was

Table 1. Plasmids used

Plasmid	Vector	<i>unc</i> genes present	Reference
pACYC184	–	None	Chang & Cohen (1978)
pAN36	pACYC184	<i>uncDC</i>	Downie <i>et al.</i> (1980)
pAN45	pACYC184	<i>uncBEFHAGDC</i>	Downie <i>et al.</i> (1980)
pRPG32	pACYC184	<i>uncDC</i>	Gunsalus <i>et al.</i> (1982)
pUC118	–	None	Vieira & Messing (1987)
pDP31	pUC118	<i>uncDC</i>	Parsonage <i>et al.</i> (1987b)

Table 2. *E. coli* strains used

Strain	Genotype	Reference
AN818	<i>uncD409, argH, pyrE, entA, recA</i>	Cox <i>et al.</i> (1978)
AN869	<i>unc418::Mu B⁺E⁺F⁺H⁺A⁺G⁺D⁺C⁻ argH, pyrE, entA, recA</i>	Gibson <i>et al.</i> (1978)
AN1272	<i>unc420::Mu B⁺E⁺F⁺H⁺A⁺G⁺D⁺C⁻ argH, pyrE, entA, recA</i>	Downie <i>et al.</i> (1981)
DP6	$\Delta(\textit{uncB-C})\textit{ilv}::\text{Tn10, recA1, endA1, gyrA96, thi, hsdR17, supE44, relA1, } \Delta(\textit{lac-proAB}), [\text{F}', \textit{traD36, proAB, lacI}^q\text{ZAM15}]$	Parsonage <i>et al.</i> (1988)

precipitated with ethanol and redissolved in 10 mM-Tris/HCl/1 mM-EDTA, pH 8.0.

Screening of mutagenized plasmids for mutant *uncD* alleles

Mutagen-treated plasmid was used to transform strain AN818 (*uncD409, recA*) and transformed cells on LB plates were replicated to glucose and succinate media to identify colonies showing an Unc⁻ phenotype, i.e. slow growth on non-fermentable substrate (Cox & Downie, 1979). Plasmid minipreps were made from the Unc⁻ colonies after growing up in 5 ml of LB/chloramphenicol medium, and were used to transform strain AN869 (*uncC⁻*) and AN1272 (*uncD⁻C⁻*). Phenotypic screening by assay of growth on succinate and glucose plates was repeated and those plasmids which complemented strain AN869 but did not complement strain AN1272 were retained. Where the parent plasmid was pRPG32 or pAN36, these tests were sufficient to establish that the final plasmids contained a mutant *uncD* allele whereas they expressed a normal *uncC* gene. Where the parent plasmid was pAN45 it was necessary to perform a further round of complementation tests on succinate plates by transforming strains containing reference mutant *uncB, E, F, H, A* and *G* alleles to be sure that only the *uncD* gene on the mutagenized plasmid was abnormal.

Subcloning procedures for DNA sequencing of mutagenized plasmids

The 0.9 kb *Pst*I fragment [containing the distal part of *uncG* and codons 1–280 of *uncD* (the *E. coli uncD* gene has 460 codons; the *N*-terminal amino acid of mature β -subunit corresponds to codon 2)] and the 1.2 kb *Pst*I fragment (containing codons 280–460 of *uncD* and the whole of *uncC*) were obtained by *Pst*I-digestion of the *uncD* mutant plasmids and inserted into *Pst*I-digested pUC118. After preparation of single-stranded DNA, sequencing of the entire *uncD* gene was performed using M13 universal primer and a series of synthetic 20-mer primers (Parsonage *et al.*, 1987a).

Other procedures

Growth of bacterial cells, preparation of cell membrane vesicles, measurement of growth yield on limiting glucose medium, enzymes and chemicals, agarose gel electrophoresis, cell transformations, plasmid DNA preparations, preparation of single stranded DNA and DNA sequencing by the dideoxy method were as described by Parsonage *et al.* (1987a,b). Strain DP6 was used as host for single-stranded DNA preparations to prevent incorporation of wild-type *unc* DNA from host into phage DNA (Parsonage *et al.*, 1988).

Purification of soluble F₁-ATPase was as described by Duncan & Senior (1985). Purified soluble F₁ protein was assayed by Bradford's method (Bradford, 1976).

Measurement of pH gradient formation induced by NADH or ATP in membrane vesicles was as described by Perlin *et al.* (1983), utilizing Acridine Orange fluorescence measurement and 0.5 mg of protein/ml in each case. Membrane protein was estimated by the method of Lowry *et al.* (1951).

Assay of ATPase activity was as described by Wise *et al.* (1983). pH-dependence of ATPase activity was as described by Senior *et al.* (1983).

RESULTS

Isolation of new mutant *uncD* alleles

As described in the Materials and methods section, random mutagenesis of plasmids by nitrosoguanidine or hydroxylamine followed by phenotypic screening for slow growth on non-fermentable substrate (succinate) was used to isolate mutant *uncD* alleles. DNA sequencing was then carried out to identify the actual base changes present. Ignoring duplicate mutations, this resulted in identification of six new missense F₁- β -subunit mutations, five of which are previously unreported, and one of which, though reported previously in the literature as an assembly-defective mutant, appeared here to have properly-assembled F₁ (see later). The actual DNA and amino acid sequence changes found are listed in Table 3.

Table 3. DNA sequence and amino acid sequence changes in *uncD* (β -subunit) mutants

Mutant plasmid	Parent plasmid	Codon change	Amino acid change
pAN216*	pAN36	Codon 138, TGT → TAT	Cys-137 → Tyr
pAN122*	pAN45	Codon 143, GGC → GAC	Gly-142 → Asp
pDP2†‡	pRPG32	Codons 147, GGT → AGT and 208, GGC → GAC	Gly-146 → Ser Gly-207 → Asp
pDP3†	pRPG32	Codon 208, GGC → GAC	Gly-207 → Asp
pDP4†	pRPG32	Codon 224, GGT → GAT	Gly-223 → Asp
pAN215*	pAN36	Codons 404, CCG → TCG and 416, GGT → GAT	Pro-403 → Ser Gly-415 → Asp

* Mutagenized with nitrosoguanidine.

† Mutagenized with hydroxylamine.

‡ Codon 399 was also found to be changed (CGC → CGT) but this does not change residue 398 (Arg).

Table 4. Growth characteristics of mutant strains

Glucose medium contained chloramphenicol or ampicillin as appropriate. Results shown are means of at least quadruplicate determinations.

Plasmid/host strain	Genotype	Growth on succinate plates	Growth yield on limiting (3 mM) glucose (A_{590})
pRPG32/AN1272	<i>unc</i> ⁺	+++	0.85
pACYC184/AN1272	<i>unc</i> ⁻	—	0.40
pAN216/AN1272	β Tyr-137	+	0.56
pAN122/AN1272	β Asp-142	+	0.57
pDP2/AN1272	β Ser-146, β Asp-207	+	0.49
pDP3/AN1272	β Asp-207	+	0.55
pDP4/AN1272	β Asp-223	+	0.58
pAN215/AN1272	β Ser-403, β Asp-415	+	0.58
pDP31/AN1272	<i>unc</i> ⁺	+++	0.85
pUC118/AN1272	<i>unc</i> ⁻	—	0.40
pDP2R/AN1272	β Ser-146	+	0.70

It is important to stress that the entire *uncD* gene was sequenced in each case, and was normal except for the mutations listed in Table 3.

Attempts to separate the double mutations in plasmids pDP2 and pAN215

Plasmid pDP2 contained missense mutations in *uncD* codons 147 and 208. These were successfully separated as follows. Plasmid pDP2 was digested with *Hind*III and *Sst*I (the latter cleaves at codon 163 of *uncD*) and the 0.9 kb fragment including *uncD* codons 1–163 (with mutant codon 147) was isolated. Plasmid pDP31 (see Table 1) was digested with *Sst*I and *Kpn*I, liberating a 1.6 kb fragment containing codons 164–460 of *uncD* (with normal codon 208), which was isolated. Plasmid pDP31 was also digested with *Kpn*I and *Hind*III, and the 3.2 kb vector-containing fragment was isolated. The three isolated fragments were then ligated together, and the mixture was used to transform strain AN869, selecting for growth on succinate/ampicillin plates. DNA sequencing was performed to prove that the resultant reconstructed plasmid (named pDP2R) contained the required mutation in codon 147 and a normal codon 208. Plasmid pDP2R is therefore a mutated equivalent of pDP31. Plasmid pDP3 contains the codon 208 mutation alone (Table 3).

Plasmid pAN215 contained mutations in codons 404 and 416. Unfortunately, we could find no useful intervening restriction endonuclease site for separation of the two mutations. We attempted, by plating out suspensions of cells on succinate media containing varied amounts of casamino acids and looking for colonies showing improved growth, to identify revertants in which one or other of the mutant codons had reverted to normal. This approach also was unsuccessful. However, since the mutations are closely spaced, the effects seen may be related to at least a small region of the protein (residues 403–415), although not to a specific residue.

Characterization of the mutations obtained

For biochemical characterization of the mutations, strain AN1272 (*uncD*⁻ C⁻) was transformed with each of the mutant plasmids.

Effects on cell growth. Growth of the mutant strains on succinate plates, and growth yields on limiting glucose, are shown in Table 4. None of the mutations gave a full *Unc*⁻ phenotype; rather, impairment of cell growth was partial. Thus ATP synthesis by oxidative phosphorylation was partly but not totally inhibited in each case.

Membrane ATPase activity. Membrane ATPase

Table 5. ATPase activities in *uncD* mutants

Results shown are means of at least triplicate assays. Normal (*unc*⁺) purified F₁-ATPase specific activity was 24 μ mol of P_i released/min per mg. Soluble F₁-ATPase from mutant Gly-146 $\rightarrow$ Ser was $\sim$ 50 % pure as judged by SDS gel electrophoresis. A low yield of soluble F₁ was obtained in this case, preventing purification to homogeneity.

Genotype	Membrane ATPase (% of normal)	Purified soluble F ₁ ATPase (% of normal)
<i>unc</i> ⁺	100	100
<i>unc</i> ⁻	0	—
Cys-137 $\rightarrow$ Tyr	12	10
Gly-142 $\rightarrow$ Asp	7.1	13
Gly-146 $\rightarrow$ Ser and Gly-207 $\rightarrow$ Asp	8.7	Not determined
Gly-146 $\rightarrow$ Ser	61	33
Gly-207 $\rightarrow$ Asp	6.1	8.3
Gly-223 $\rightarrow$ Asp	21	32
Pro-403 $\rightarrow$ Ser and Gly-415 $\rightarrow$ Asp	26	29

activities are shown in Table 5 (column 1) and spanned a wide range of activities (6–61 % of normal). In these experiments normal (*unc*⁺) membrane ATPase activity was $\sim$ 1–1.6 μ mol of ATP hydrolysed/min per mg. An appropriate *unc*⁺ control strain was run alongside all mutant preparations so that mutant membranes and *unc*⁺ membranes were prepared under exactly the same conditions of medium, shaking rate, temperature, etc.

Purified soluble F₁-ATPase activity. These activities are shown in Table 5 (column 2). A range of specific activities was noted. In each case, the F₁-ATPase eluted from the Sephacryl S-300 column during the terminal purification step in a normal elution volume, showing that the molecular size of the F₁ (382000 Da) was unaffected. SDS/polyacrylamide gel electrophoresis of the purified F₁-ATPase showed, in each case, the presence of α , β , γ , δ and ϵ subunits in apparent normal stoichiometry. Thus, we may conclude that in each case shown in Table 5 (column 2) an F₁-ATPase of normal molecular size and subunit structure was assembled in the cell, and that each of the mutations affected catalytic turnover rate.

Stability of purified mutant F₁-ATPase enzymes. Two simple methods which have proved useful for studying the oligomeric subunit stability of F₁-ATPases are pH-dependence of ATPase activity (e.g. Wise *et al.*, 1983) and cold lability of ATPase activity (e.g. Rao *et al.*, 1988b). These tests were applied to each of the purified mutant F₁-ATPase enzymes.

The mutation β Gly-142 $\rightarrow$ Asp caused substantial loss of activity at higher pH values (Fig. 1). Previously (Wise *et al.*, 1983; Parsonage *et al.*, 1987a) it was seen that the mutation β Gly-142 $\rightarrow$ Ser caused a similar effect, which was shown by gel filtration tests to be due to dissociation of F₁ into subunits at elevated pH. A similar explanation seems likely for the β Gly-142 $\rightarrow$ Asp mutant. The mutation β Gly-207 $\rightarrow$ Asp also caused strong impairment of activity at higher pH, and the double mutant

Fig. 1. pH dependence of ATPase activity of purified mutant F₁

●, *unc*⁺; □, β Gly-207 $\rightarrow$ Asp; △, double mutant β Pro-403 $\rightarrow$ Ser, β Gly-415 $\rightarrow$ Asp; ○, β Gly-223 $\rightarrow$ Asp; ◇, β Cys-137 $\rightarrow$ Tyr; ▲, β Gly-146 $\rightarrow$ Ser; ▽, β Gly-142 $\rightarrow$ Asp. A single line was drawn through data for mutants β Asp-223, β Tyr-137 and β Ser-146; hence only five lines are shown even though the profiles of seven enzymes are presented.

β Pro-403 $\rightarrow$ Ser, β Gly-415 $\rightarrow$ Asp was affected to a lesser extent. The other three mutations showed similar-to-normal profiles (Fig. 1). None of the mutations seriously affected the cold-lability of the enzyme (results not shown).

pH gradient formation in membrane vesicles. The percentage quenching of Acridine Orange fluorescence in membrane vesicles was assayed as a measure of NADH- and ATP-induced pH gradient formation (see Materials and methods section). NADH-induced pH gradient formation was unaffected by the mutations. Both the normal and mutant membranes showed 80–85 % quenching of fluorescence on addition of NADH, indicating that the mutant membranes retained normal impermeability to protons. ATP induced 80 % quenching of fluorescence in normal membranes. This was reduced to greater or lesser extent in each of the mutants (results not shown) in accordance with the severity of the inhibition of ATPase (Table 5). However, considerable remaining ATP-driven proton-pumping was noted in each case, consistent with the fact that none of the mutations caused a drastic reduction of membrane-ATPase. These results confirmed that in each of the mutants, F₁ was assembled on the membrane and that catalytic turnover on F₁ was impaired.

DISCUSSION

General

The procedure of random chemical mutagenesis of plasmid-encoded *uncD* gene yielded six mutant *uncD* alleles, all of which allowed assembly of an F₁-ATPase *in vivo* which evinced impairment of ATP synthesis, ATPase activity and ATP-driven proton-pumping. Purified soluble F₁-ATPase could be obtained in all cases. [In the case of the β Gly-146 $\rightarrow$ Ser mutant, carried on plasmid pDP2R, only $\sim 50\%$ pure soluble F₁ was obtained, but it may well be possible to obtain pure soluble F₁ from this mutant after sub-cloning the mutant *uncD* allele into a plasmid such as pAN45, which overexpresses F₁F₀ (Perlin *et al.*, 1983).]

Comments on mutations obtained

The catalytic nucleotide-binding domain of the β -subunit of *E. coli* F₁-ATPase has been predicted by our laboratory to be formed by residues 140–335 approximately (Duncan *et al.*, 1986; Senior, 1988). A diagram of this predicted domain is shown in Fig. 2. Five of the mutations generated in this study lie within, or very close to, this predicted domain as shown in Fig. 2 (i.e. mutations affecting residues 137, 142, 146, 207, 223). It is likely that these mutations impair catalysis by changing the interaction between the catalytic nucleotide-binding domain and bound substrates, products or transition states. The degree of conservation of these residues in β -subunits from 21 different species is shown in Table 6.

Individual comments on some of these mutations may

Fig. 2. The location of five mutations in or close to the predicted catalytic nucleotide-binding domain of β -subunit

In the model, arrows represent β -strands (1–5), and cylinders represent α helices (A–E). Bound ATP is shown in black. See Duncan *et al.* (1986) for further details.

Table 6. Conservation of residues in 21 β -subunit sequences

Residue	Amino acid in <i>E. coli</i>	Amino acid found among 21 species
137	Cys	17 Ala, 1 Glu, 2 Cys, 1 Thr
142	Gly	21 Gly
146	Gly	21 Gly
207	Gly	21 Gly
223	Gly	14 Gly, 7 Ala
403	Pro	19 Pro, 1 Lys, 1 Thr
415	Gly	21 Gly

be made as follows. Gly-142 $\rightarrow$ Asp is the second mutation obtained at this position, the previous one being *uncD*412 (Gly-142 $\rightarrow$ Ser) which was characterized in detail by Duncan & Senior (1985). Gly-146 $\rightarrow$ Ser lies in predicted β -strand 1 of the catalytic nucleotide-binding domain, which immediately precedes the highly-conserved 'glycine-rich flexible loop' region discussed by Fry *et al.* (1986). Gly-207 $\rightarrow$ Asp is close to a previous, highly inhibitory mutation found at residue-209 (*uncD*484, Met-209 $\rightarrow$ Ile) which was characterized in detail by Duncan & Senior (1985). Gly-223 $\rightarrow$ Asp was isolated previously in a haploid chromosomal mutant strain and was reported to cause defective assembly of F₁-ATPase (Noumi *et al.*, 1986). In our work the mutation was carried on a plasmid, and assembly of F₁-ATPase of normal molecular size and subunit structure did occur *in vivo*. This further demonstrates that isolation and expression of random *uncD* mutations on plasmids, as described here, is a valuable approach. Previous work of Jans *et al.* (1983, 1984) and Kumamoto & Simoni (1986) has shown that increased gene dosage facilitates assembly of F₁F₀-ATPase containing mutant subunits *c* or *b*. It may be that the reason our recent site-directed mutagenesis studies have not been hampered by defective assembly of F₁-ATPase *in vivo* is that those mutations too are plasmid-borne and -expressed (Parsonage *et al.*, 1987b).

The other mutant *uncD* allele generated in this work was a double mutant Pro-403 $\rightarrow$ Ser, Gly-415 $\rightarrow$ Asp. These residues lie outside the predicted catalytic nucleotide-binding domain. Interestingly, the reactive nucleotide analogue *p*-fluorosulphonylbenzoyl-5'-adenosine (FSBA) has been shown to react at low pH with residue His-427 of beef-heart mitochondrial F₁ β -subunit, which is equivalent to residue Ser-413 of *E. coli* F₁ β -subunit (Bullough & Allison, 1986). These workers suggest that this residue probably does not have a direct catalytic role. This leads us to offer the following speculation. Since FSBA apparently binds in the non-catalytic nucleotide binding sites of F₁, which reside on the α -subunits (Senior, 1988; Rao *et al.*, 1988a), the labelling of residue β His-427 (residue β Ser-413 in *E. coli*) by FSBA suggests that residue β Ser-413 in *E. coli* abuts upon the α -subunit nucleotide-binding domain. Thus the double mutation at residues 403,415 may be affecting an α - β intersubunit contact region which is important for normal catalysis. Directed mutagenesis of this region may be useful to substantiate or rebut this speculation. It is interesting to note that residue Pro-403 is conserved in 19 of 21 species for which the β -subunit sequence is now known, and residue Gly-415 is conserved in all 21 species (Table 6).

Conclusions

Generation of mutant *uncD* alleles on plasmids by random mutagenesis appears to be a valuable approach which should allow many more F_1 - β -subunit mutations to be obtained, without the associated problem of defective assembly of F_1 -ATPase seen previously when β -subunit mutations were isolated in the chromosomal *uncD* gene. This will facilitate molecular studies of the mechanism of catalysis in F_1 -ATPase. Six new mutants obtained by this approach are reported here. Each of the six displays interesting features meriting more detailed study.

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