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Amino acid substitutions in the ε-subunit of the F_1F_0-ATPase of *Escherichia coli*

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A mutant strain of *Escherichia coli* was isolated in which Gly-48 of the mature ε-subunit of the energy-transducing adenosine triphosphatase was replaced by Asp. This amino acid substitution caused inhibition of ATPase activity (about 70%), loss of ATP-dependent proton translocation and lowered oxidative phosphorylation, but did not affect proton translocation through the F_0. Purified F_1-ATPase from the mutant strain bound to stripped membranes with the same affinity as the normal F_1-ATPase. Partial revertant strains were isolated in which Pro-47 of the ε-subunit was replaced by Ser or Thr. Pro-47 and Gly-48 are predicted to be residues 2 and 3 in a Type II β-turn and the Gly-48 to Asp substitution is predicted to cause a change from a Type II to a Type I or III β-turn. Space-filling models of the β-turn (residues 46–49) in the normal, mutant and partial revertant ε-subunits indicate that the peptide oxygen between Pro-47 and Gly-48 is in a different position to the peptide oxygen between Pro-47 and Asp-48 and that the substitution of Pro-47 by either Ser or Thr restores an oxygen close to the original position. It is suggested that the peptide oxygen between Pro-47 and Gly-48 of the ε-subunit is involved either structurally in inter-subunit H-bonding or directly in proton movements through the F_1-ATPase.

Introduction

The F_1F_0-ATPase enzyme complex catalyses the terminal step in oxidative phosphorylation and is located in mitochondrial, chloroplast and bacterial membranes. The structure is apparently highly conserved and the complex can be readily dissociated into two portions, the water-soluble F_1-ATPase and the membrane-bound F_0 portion which forms a proton pore. The *Escherichia coli* F_1-ATPase comprises five subunits (α, β, γ, δ, ε) and the F_0 sector three subunits (*a*, *b*, *c*) (see Ref. 1). The ε-subunit of the *E. coli* F_1-ATPase has been purified and characterised as a globular protein of molecular weight 15000–16000 with an α-helical content of about 40% [2]. The ATPase activity of purified F_1-ATPase was inhibited by the addition of purified ε-subunit but the addition of ε to the membrane-bound F_1F_0-ATPase complex did not inhibit ATPase activity [2]. The ε-subunit has also been shown to form a specific, high-affinity equimolar complex with the γ-subunit and ε binds to the F_1-ATPase through interactions with the γ-subunit [3].

In *E. coli* the genes coding for the subunits of the F_1F_0-ATPase complex form the *unc* operon at about minute 83.5 on the chromosome and the complete DNA sequence has been determined (see Ref. 4). Mutations have been described in each of the genes of the *unc* operon and considerable

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information has been gained from the study of these mutant strains with regard to assembly, structure, and function (see Ref. 1). Only three mutations, none of them involving single amino acid substitutions, affecting the ε-subunit have been described. In two of these mutant strains the ε-subunit was not formed [5,6], while in the third a small amount of normal ε-subunit was formed (this laboratory, unpublished results). In the present paper we describe a mutation in the *uncC* gene encoding the ε-subunit and two partial revertant strians each carrying an additional mutation in the same gene.

Materials and Methods

Enzymes and chemicals. Restriction endonucleases and T4-virus DNA ligase were obtained from Amersham Australia Pty. Ltd. All chemicals used were of the highest quality available. α-Labelled [^{32}P]ATP was obtained from Amersham (Australia) Pty. Ltd, LMP agarose from Bethesda Research Laboratories (U.S.A.) and dideoxynucleotides and deoxynucleotides from Boehringer Mannheim Australia. The space-filling models were constructed with CPK Precision Molecular Models.

Bacterial strains and plasmids. All of the bacterial strains used were derived from *E. coli* K12 and are described, together with the plasmids used, in Table I.

Genetic techniques. The techniques used for genetic experiments were as outlined previously [5,11,12].

Preparation of plasmids. Plasmid DNA was prepared as described by Selker et al. [13]. Chromosomal DNA was prepared using the method described by Saito and Miura [14].

DNA sequencing. DNA sequences were determined using the dideoxynucleotide chain termination method as described by Sanger [15], using deoxy (α-[^{32}P]ATP), and the universal 17-mer M13 sequencing primer [16] or an *uncC*-specific 20-mer primer ($^{5'}$GTTTCGTTCCATGGCTCGC$^{3'}$).

Media and growth of organisms. The mineral-salts minimal medium used and the additions were as described previously [17]. Cells for the preparation of membranes were grown in 14-l fermenters essentially as described previously [18]. The media in the fermenters was supplemented with 5% (v/v) Luria broth [19]. Turbidities of cultures were measured with a Klett-Sumerson colorimeter.

Preparation of cell membranes. The preparation and treatment of membranes were as previously described [20].

Two-dimensional gel electrophoresis. The two-dimensional gel electrophoresis technique was essentially that of O'Farrell [21,22] involving isoelectric focussing in the first dimension and sodium dodecyl sulphate polyacrylamide-gradient-gel electrophoresis in the second dimension.

TABLE I

STRAINS OF *ESCHERICHIA COLI* AND PLASMIDS USED

Bacterial strain or plasmid	Relevant genotype	Notes and references
AN346	*argH ilvC pyrE entA*	*unc*$^+$ parent strain
AN1510	*uncC*473 *argH pyrE entA*	described in this paper
AN1518	*uncC*473 *argH pyrE entA recA*	described in this paper
AN2540	*uncC*538 *argH pyrE entA*	partial revertant of AN1510
AN2618	*uncC*543 *argH pyrE entA*	partial revertant of AN1510
AN2620	*uncC*544 *argH pyrE entA*	partial revertant of AN1510
AN818	*uncD*409 *argH pyrE entA recA*	7
AN2387	pAN337 *uncC*473/*uncC*473 *argH pyrE entA recA*	described in this paper
AN869	Mu : : *uncC argH pyrE entA*	5
pHC79	Ap^R Tc^R	8
PBR328	Cm^R Tc^R Ap^R	9
M13 mp18		10
pAN337	*uncC*473 Cm^R	described in this paper

Other methods. ATPase and atebrin fluorescence-quenching activities were assayed as described previously [12]. One unit of ATPase activity is taken as the hydrolysis of 1 μmol ATP per min at 30°C and specific activities are expressed as units/mg protein. Protein concentrations were determined using Folin's phenol reagent [23]. F_1-ATPase binding to stripped membranes was measured as described previously [24].

Results

Isolation and genetic characterization of the mutant uncC473 allele and partial revertants

The *uncC*473 allele was generated by *N*-methyl-*N'*-nitro-*N'*-nitrosoguanidine treatment and transferred from the original isolate into strain AN346 by bacteriophage P1-mediated cotransduction with *ilvC*. The transductant (AN1510) grew poorly on succinate-minimal medium and had a growth yield on limiting concentrations of glucose even lower than is characteristic of a strain in which electron transport is uncoupled from ATP synthesis (see below). Partial diploid strains were prepared by mating a *recA* derivative (AN1518) of strain AN1510 with donor strains each carrying a mutant allele of the *uncA, uncB, uncC, uncD, uncE, uncF, uncG* or *uncH* genes on F-plasmids. Complementation, as judged by growth on succinate of the partial diploid strains, was obtained between the *uncC*473 allele and each of the mutant alleles tested with the exception of mutant alleles of the *uncC* gene. Spontaneous revertants from strain AN1510(*uncC*473) were also isolated after plating on minimal medium with succinate as energy source. Three such strains were purified viz. AN2540, AN2618 and AN2620.

The nucleotide sequences of the uncC473 and revertant alleles

Chromosomal DNA was prepared from strain AN1510(*uncC*473) and the revertant strains AN2540, AN2618 and AN2620. The DNA was then partially digested with the restriction endonuclease *Hin*dIII and ligated to *Hin*dIII-digested cosmid vector pHC79. The concatamers of appropriate size were then packaged in vitro into bacteriophage λ particles. Strain AN818 (*uncD*409) was transformed with each of the λ-packaged DNA preparations and selection made for transformants on ampicillin-succinate minimal medium. One colony was selected and purified from each of the group of transformants derived from the four DNA preparations. Plasmid DNA

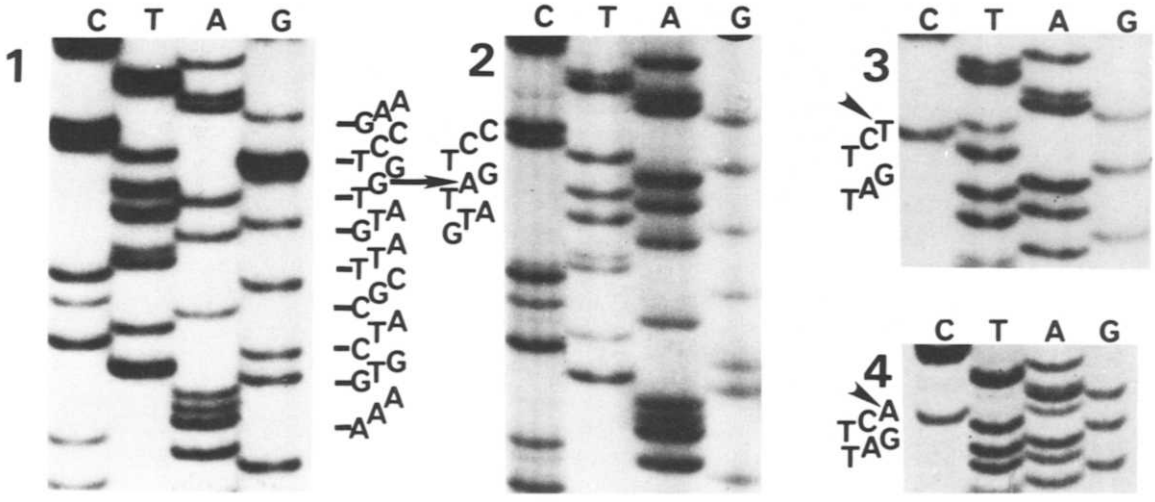

Fig. 1. Portion of DNA-sequencing gels of DNA derived from a normal strain (1); strain AN1510(Gly-48 → Asp) (2); strain AN2540 (Gly-48 → Asp;Pro-47 → Ser) (3) and strain AN2618(Gly-48 → Asp;Pro47 → Thr) (4). The sequence of the corresponding region of DNA derived from AN2620(Gly-48 → Asp;Pro-47 → Thr) was identical with (4). The G → A change at nucleotide 146 giving the *uncC*473 allele is indicated, as are the additional base changes in the partial revertant strains.

was prepared from each of the four transformants. The Pst fragments carrying the mutant or revertant *uncC* alleles were purified by electrophoresis in low melting point agarose and cloned into the plasmid pBR328 and the M13 vector mp18.

The nucleotide sequences of the four *uncC* alleles were determined from the single-stranded forms of the mp18 clones using the universal M13 oligonucleotide primer and an *uncC* gene-specific oligonucleotide primer. The nucleotide sequences of all four alleles differed from normal in that a G → A base change occurred at nucleotide 146 (Fig. 1). Gly at position 48 in the normal mature ε-subunit was therefore replaced by Asp in the mutant and this amino acid substitution also occurred in the three revertant strains. The *uncC*538 allele had, in addition, a C → T base change at nucleotide 142 and both *uncC*543 and *uncC*544 alleles had additional C → A base changes also at nucleotide 142 (Fig. 1). The corresponding ε-subunits therefore differed from normal in two positions; Pro-47 is replaced by Ser (*uncC*538) or Thr (*uncC*543, *uncC*544) and Gly-48 is replaced by Asp.

Properties of strains carrying the uncC473, uncC543, or uncC538 alleles

Strain AN1518(Gly-48 → Asp) * grew slowly on succinate-minimal medium (Fig. 2) and had a growth yield (125 Klett units) on limiting concentrations of glucose (5 mM), lower than that obtained for a typical uncoupled strain (140 Klett units). The growth yield for a normal strain was 240 Klett units. Strain AN1518(Gly-48 → Asp) was transformed with plasmid pAN337 (a derivative of pBR328 carrying the *uncC*473 allele) to give strain AN2387(pAN337,Gly-48 → Asp). This strain grew better on succinate minimal medium than AN1518 (Fig. 2) and also had a growth yield (150 Klett units) on limiting concentrations of glucose which was higher than for AN1518. The two revertant strains AN2540(Gly-48 → Asp;Pro47 → Ser) and AN2618(Gly-48 → Asp;Pro47 → Thr) both grew well on succinate-minimal medium and had similar growth yield values of about 190 Klett units.

* The amino acid substitution(s) in the ε-subunit of the F_1-ATPase in mutant strains are indicated where appropriate.

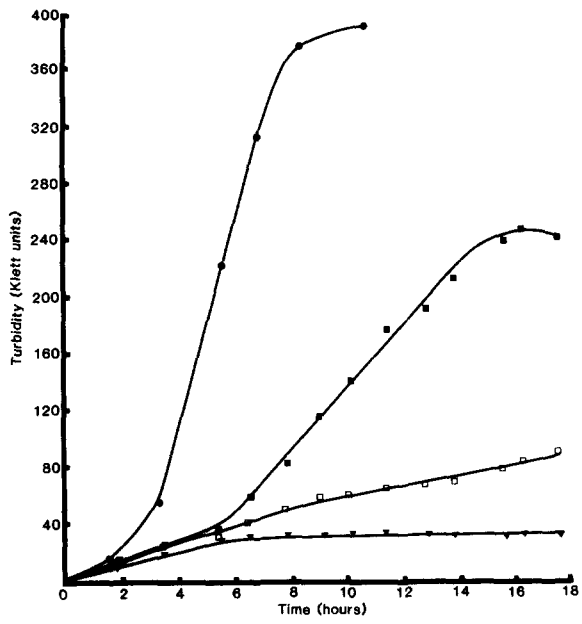

Fig. 2. Growth on succinate minimal medium of normal and mutant strains. ●——●, strain AN346(*unc*$^+$); ■——■, strain AN2387(pAN337,Gly-48 → Asp); □——□, strain AN1518(Gly-48 → Asp); ▼——▼, strain AN869(Mu::*uncC*).

Membranes were rpepared from strains AN 1518(Gly-48 → Asp), AN2387(pAN337,Gly-48 → Asp), the two partial revertant strains AN2540 (Gly-48 → Asp;Pro-47 → Ser) and AN2618(Gly-48 → Asp;Pro47 → Thr) and a control strain AN346. The membranes were examined for ATPase activity and for NADH- and ATP-dependent atebrin fluorescence quenching activity (Fig. 3). A difference was apparent between the membrane properties of the haploid strain AN1518 (Gly-48 → Asp) and strain AN2387(pAN337,Gly-48 → Pro). The membranes of strain AN1518 (Gly → Asp) were somewhat proton permeable as judged by NADH-dependent atebrin fluorescence quenching activity indicating some incomplete F_1F_0 assemblies and had an ATPase specific activity of 0.2 compared with 1.5 for the parent strain AN346. By the same criterion the presence of the multicopy plasmid carrying the *uncC*473 allele caused the formation of complete F_1F_0 assemblies (Fig. 3) with a concomitant increase in ATPase specific activity to 0.4. The ATP-dependent atebrin fluorescence quenching activity was essentially

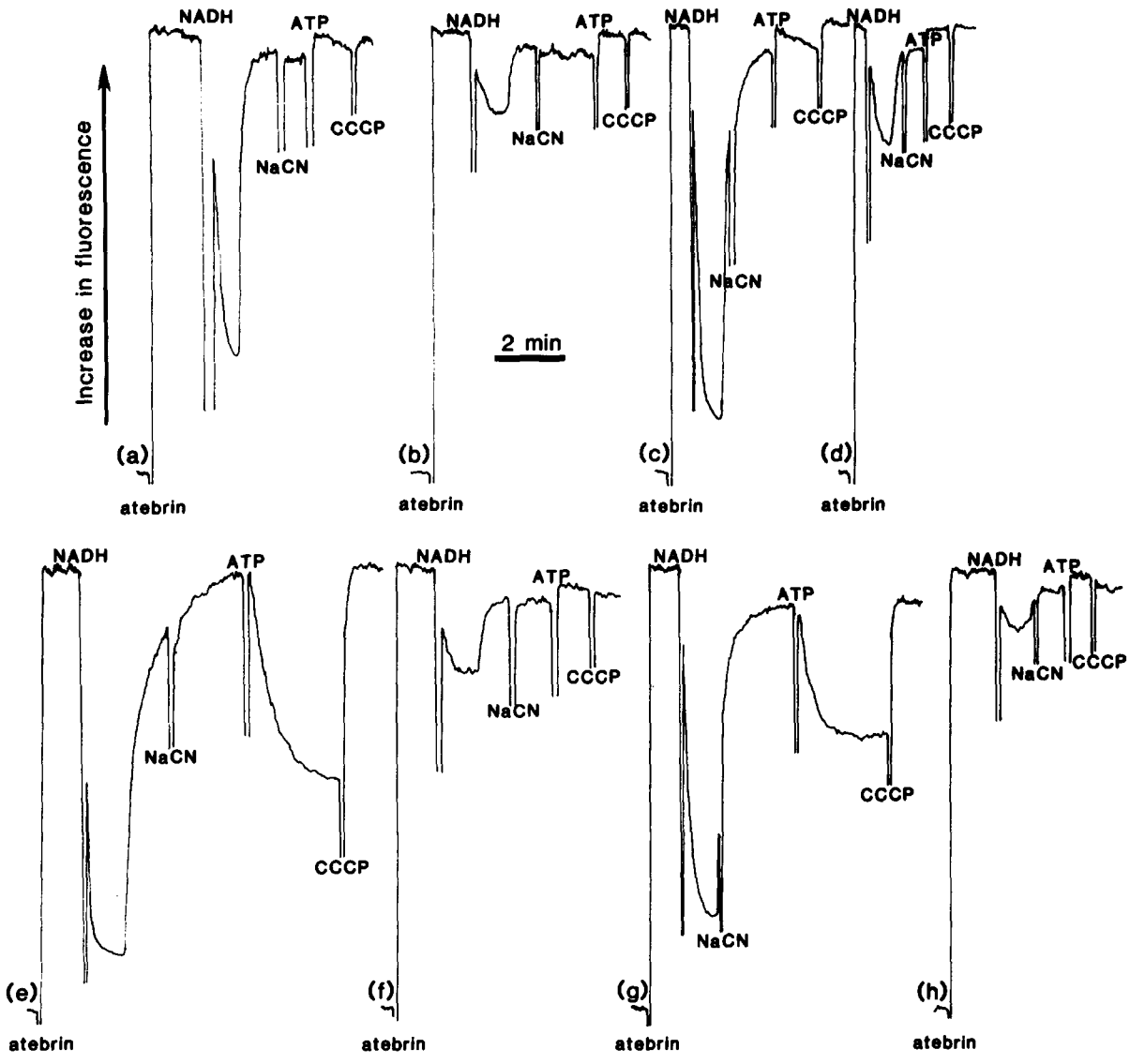

Fig. 3. Atebrin fluorescence quenching of membranes from mutant and revertant strains. Native membranes from strain AN1518(Gly-48 → Asp) (a); stripped membranes from strain AN1518 (Gly-48 → Asp) (b); native membranes from AN2387(pAN337,Gly-48 → Asp) (c); stripped membranes from AN2387(pAN337,Gly-48 → Asp) (d); native membranes from AN2540(Gly-48 → Asp;Pro47 → Ser) (e); stripped membranes from AN2540(Gly-48 → Asp;Pro47 → Ser) (f); native membranes from strain AN2618(Gly-48 → Asp;Pro47 → Thr) (g); stripped membranes from strain AN2618(Gly-48 → Asp;Pro47 → Thr) (h). Atebrin fluorescence quenching obtained with membranes from the control strain AN346 is identical to that of Fig. 5(a).

zero in both strains AN1518(Gly-48 → Asp) and AN2387(pAN337,Gly-48 → Asp) (Fig. 3) indicating that the mutation prevents ATPase-dependent proton pumping. Stripping of the F_1-ATPase from the membranes of both strains resulted in loss of NADH-dependent atebrin fluorescence quenching (Fig. 3) indicating that the F_0 had normal proton permeability.

Membranes from the two partial revertant strains had properties which differed from the parent strain and from each other indicating that the protein with the proline → serine substitution

was closer to normal than that with the proline → threonine substitution. Thus the ATPase specific activity of membranes from strain AN2540(Gly-48 → Asp;Pro47 → Ser) was 1.0 compared with 0.4 for strain AN2618(Gly-48 → Asp;Pro47 → Thr). ATP-dependent atebrin fluorescence quenching activity was about 60% of normal for membranes from strain AN2540(Gly-48 → Asp;Pro47 → Ser) compared with about 40% of normal for strain AN2618(Gly-48 → Asp;Pro47 → Thr) (Fig. 3). The membranes of strain AN2618 were also somewhat proton permeable as judged by the NADH-dependent atebrin fluorescence quenching activity.

Properties of the purified F_1-ATPase from membranes of strains AN2387, AN2540 and AN2618

The F_1-ATPase was solubilised from membrane preparations of strains AN2387(pAN337,Gly-48 → Pro), AN2540(Gly-48 → Asp;Pro47 → Ser), AN2618(Gly-48 → Asp;Pro47 → Thr) and the normal strain AN346 and purified by the method described previously [25].

The specific activities of the various preparations were determined at protein concentrations of 12 μg/ml. The F_1-ATPase from the normal strain AN346 gave linear kinetics with a specific activity of 10. However the F_1-ATPase preparations from the mutant strain AN2387 and the two partial revertant strains AN2540 and AN2618 gave low initial rates increasing with time until linearity was achieved after about 3 min. This behaviour is similar to normal F_1-ATPase preparations at lower protein concentrations, a property attributed to a slow dissociation of the ε-subunit in the presence of ATP [26]. The ε-subunits from the mutant and revertant strains therefore appeared to have a lower affinity than the normal ε-subunit, since the non-linearity was observed at a relatively high protein concentration. This property of the mutant F_1-ATPases was, however, not further investigated. Specific activities were calculated from estimated initial rates and from the final rate. For strain AN2387 F_1-ATPase the initial specific activity was 2.9 and the specific activity in the linear phase, 8.8. For the partial revertant strain AN2540 F_1-ATPase the initial specific activity was 7.8 and the specific activity in the linear phase was 19.0. The corresponding specific activities for strain AN2618 F_1-ATPase were 4.0 and 17.0.

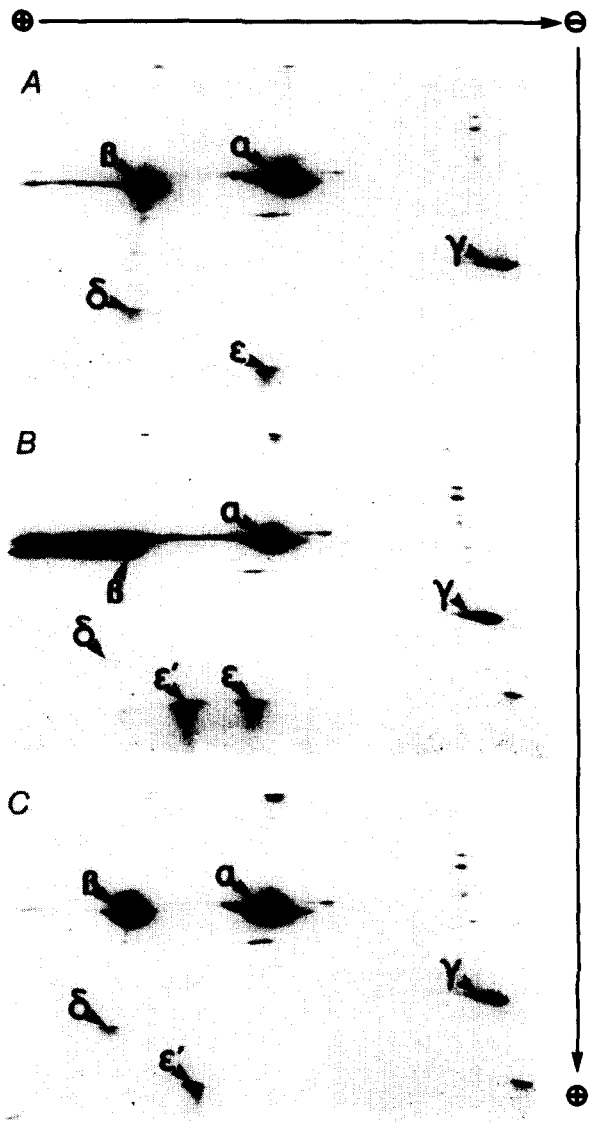

Fig. 4. Two-dimensional gel electrophoresis of purified F_1-ATPase preparations: from strain AN346(*unc*$^+$) (A); a mixture of F_1-ATPase from strains AN346(*unc*$^+$) and strain AN2387(pAN337,Gly-48 → Asp) (B); and from strain AN2387(pAN337,Gly-48 → Asp) (C). The results with purified F_1-ATPase from strains AN2540(Gly-48 → Asp;Pro47 → Ser) or strain AN2618(Gly-48 → Asp;Pro47 → Thr) were indistinguishable from (C). Samples A and C of F_1-ATPase contained about 150 μg protein. Sample B contained about 300 μg protein. In the first dimension, ampholines whose pH's ranged from 5 to 7 and from 3.5 to 10 were present at 1.2 and 0.8% (w/v), respectively. In the second dimension, an acrylamide gradient of 10.5 to 24.5% (w/v) was used. The positions of the α, β, γ, δ and ε-subunits are indicated as is that of the mutant ε-subunit, ε^1.

The F_1-ATPase preparations were examined by two-dimensional gel electrophoresis (Fig. 4). The ε-subunit of the F_1-ATPase from the mutant strain AN2387 and the two partial revertant strains AN2540 and AN2618 had isoelectric points of greater acidity than a normal ε-subunit (Fig. 4).

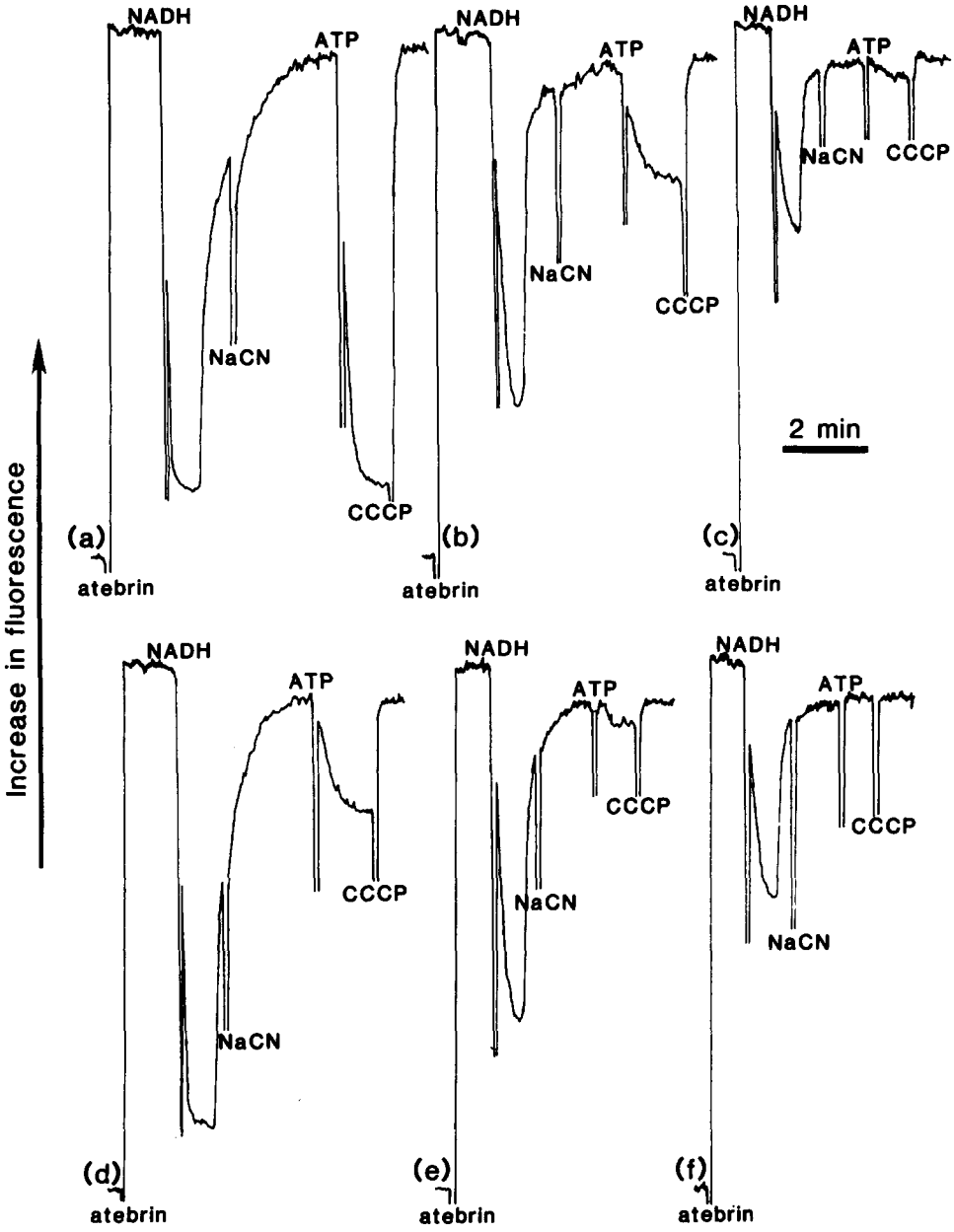

Fig. 5. Atebrin fluorescence quenching of reconstituted membranes. To stripped membranes (about 0.5 mg protein) from strain AN346(*unc*$^+$) was added (a) (b) (c) about 100 μg, 50 μg or 25 μg, respectively, of purified F_1-ATPase from strain AN346(*unc*$^+$) or (d) (c) (f) about 100 μg, 50 μg or 25 μg, respectively of purified F_1-ATPase from strain AN2540(Gly-48 → Asp;Pro47 → Ser). Essentially the same results were obtained as in (d) (e) and (f) with the F_1-ATPase preparation from strain AN2387(pAN337,Gly-48 → Asp). Saturation of the stripped membranes required the addition of 200 μg F_1-ATPase from strain AN2618(Gly-48 → Asp;Pro47 → Thr) although the maximum ATP-dependent atebrin fluorescence quenching was also 20%.

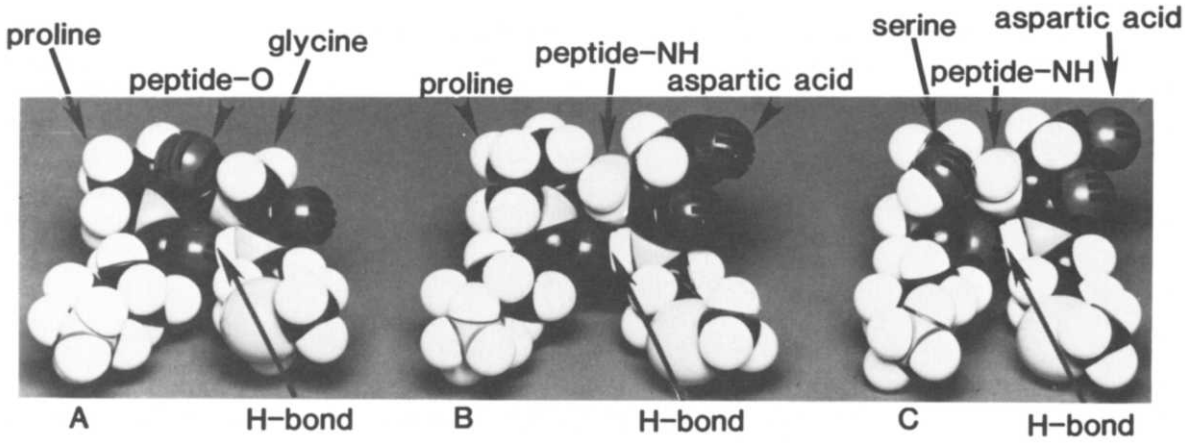

Fig. 6. Space-filling models of residues 46–49 of the ε-subunit from *E. coli* F_1-ATPase from strain AN346(*unc*$^+$) (A); strain AN1510(Gly-48 → Asp) (B) and strain AN2540(Gly-48 → Asp;Pro47 → Ser) (C). Residues 46 and 49 are lysine and methionine, respectively, in each model.

The F_1-ATPases from the mutant strain and the partial revertant strain AN2540(Gly-48 → Asp;Pro47 → Ser) bound to stripped membranes with the same affinity as normal F_1-ATPase both with regard to the binding of F_1-ATPase to stripped membranes (data not shown) and in the reconstitution of NADH- and ATP-dependent atebrin fluorescence quenching activities (Fig. 5). A surprising aspect of the reconstituted membranes was the similar ATP-dependent atebrin quenching activity obtained for the mutant AN2387 F_1-ATPase and the revertant strain AN2540 F_1-ATPase (15–20%) when the membranes from which the F_1-ATPases were prepared had ATP-dependent atebrin fluorescence quenching activities ranging from less than 5% (for strain AN2387) to about 50% (for strain AN2540). The F_1-ATPase from strain AN2618 differed from the F_1-ATPase preparations from the other strains in that it bound with a lower affinity to stripped membranes (data not shown).

Molecular modelling

The amino acid sequence Lys-Pro-Gly-Met at positions 46–49 in the ε-subunit has been predicted to constitute a β-turn [27]. With proline and glycine in positions 2 and 3 of the turn there is a high probability that residues 46–49 form a Type II β-turn [28]. Space-filling models were used to construct such a turn (Fig. 6). When glycine is replaced by aspartic acid, while a β-turn remains likely, a Type II turn is not feasible due to the bulkiness of the aspartic acid side-chain by comparison with glycine. A change to a Type I or Type III turn results in the CO and NH of the peptide linkage between proline and aspartic acid flipping over (Fig. 6). When proline is changed to a serine the turn is also likely to be a Type I or Type III β-turn and the oxygen of the serine hydroxyl can occupy a position closely similar to that occupied by the peptide oxygen in the Type II turn of the normal ε-subunit (Fig. 6).

Discussion

The substitution of Gly-48 by Asp in the ε-subunit of the F_1-ATPase results in about 70% inhibition of ATPase activity, a low level of oxidative phosphorylation as judged by growth yield values and the loss of ATP-dependent proton pumping, but does not affect the ability of the F_0 sector to translocate protons. As judged from the properties of the partial revertant strains AN2540(Gly-48 → Asp;Pro47 → Ser) and AN2618(Gly-48 → Asp;Pro47 → Thr), in which in addition to the Gly-48 to Asp substitution the Pro-47 has been replaced by serine or threonine, the effects of the original mutation are due less to the presence of the aspartic acid than to the change in conformation of the β-turn at positions 46 to 49. Comparison of space-filling models of the Type II β-turn proposed for this segment in the normal ε-subunit

with the Type I β-turns of the mutant and the two revertants suggest that the oxygen from the peptide bond between Pro-47 and Gly-48 in the normal ε-subunit and the serine or threonine hydroxyl oxygens in the revertants occupy similar positions. It would appear then that the peptide oxygen between Pro-47 and Gly-48 in the normal ε-subunit is involved either structurally in inter-subunit H-bonding or directly in proton movements through the F_1-ATPase. The change of Gly-48 to Asp effectively removes the peptide oxygen from its normal position, but this is replaced by the oxygen of the serine or threonine hydroxyl in the ε-subunit of the partial revertants. Two points of caution should be noted. Firstly, the Lys-Pro-Gly-Met sequence at positions 46–49 is not conserved in other ε-subunits [30,31] and secondly, the stucture prediction method used by Kanazawa et al. [27] is, of course, less than 100% reliable.

The difference between the ATP-dependent atebrin fluorescence-quenching activities of membranes reconstituted from the purified mutant or revertant F_1-ATPases and the membranes from which the various F_1-ATPases were prepared is striking. Thus the F_1-ATPase preparation from the Gly-48 to Asp mutant gave essentially the same ATP-dependent atebrin fluorescence quenching activity (15–20%) on reconstitution with F_1-stripped membranes as the F_1-ATPase preparation from the partial revertant strain AN2540(Gly-48 → Asp;Pro-47 → Ser). The values for the same activity in the membranes from which the F_1-ATPase was prepared ranged from less than 5% for the Gly-48 to Asp mutant to about 50% for the partial revertant strain. It would appear that on removal of the F_1-ATPase from the membranes of the mutant and the revertant strain some rearrangement occurs which restores some function to the mutant F_1-ATPase and eliminates the effect of the second amino acid substitution in the F_1-ATPase derived from the partial revertant strain. These changes also indicate that the F_1-ATPase is not likely to be assembled in the cytoplasm before interacting with an assembled F_0 in the membrane, but follows the integrated assembly pathway previously proposed for the assembly of a normal F_1F_0-ATPase complex [29].

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