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Functional stability of the *a*-subunit of the F_0F_1-ATPase from *Escherichia coli* is affected by mutations in three proline residues

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Site-directed mutagenesis was used to investigate the roles of three proline residues (Pro-103, Pro-122 and Pro-143) in the *a*-subunit of the *E. coli* F_0F_1-ATPase. All three were found to have a role in stabilizing the *a*-subunit structure in that removal of the F_1-ATPase from membranes prepared from each of the mutant strains resulted in the loss of passive proton translocation activity. Pro-103 is predicted to be within a transmembrane helix. Pro-122 and Pro-143 are located just outside the membrane and near two residues (Asp-124 and Arg-140) previously proposed to form a charge pair. The phenotype of mutants in which Pro-122 or Pro-143 were replaced by alanine was similar to previously isolated mutants affected in Asp-124 and Arg-140. This suggested that the main effect of the mutations was to destroy the charge pair between Asp-124 and Arg-140. Double mutants resulting from all possible combinations of these four mutations were constructed and, with the exception of P122A + D124A, had a similar phenotype to the single mutants. This is consistent with the idea that all four single changes had the same effect on *a*-subunit structure. In contrast, combining the P122A or P143A changes with another mutation which caused a similar phenotype (D44N) resulted in a complete loss of oxidative phosphorylation.

Introduction

The F_0F_1-ATPase enzyme complex catalyses the terminal step in oxidative phosphorylation and photophosphorylation and is located in mitochondrial, chloroplast and bacterial membranes. In *Escherichia coli* the enzyme comprises eight non-identical subunits, *a*,*b*, *c*, α, β, γ, δ and ϵ, encoded by the genes *uncB*, *F*, *E*, *A*, *D*, *G*, *H* and *C*, respectively [1]. The *a*, *b* and *c* subunits are integral membrane proteins and form the F_0 portion of the complex which can function as a proton pore. The α, β, γ, δ and ϵ subunits are peripheral membrane proteins forming the F_1-ATPase portion of the complex and which retains ATP hydrolytic activity when removed from the membrane.

The *a*, *b* and *c* subunits of the proton pore are present in a stoichiometry of 1:2:6–12 [2]. A number of approaches have been used to determine the tertiary structures of the three F_0 subunits. Proposed structures for the *b*- and *c*-subunits are well accepted [2,3], but there is no agreement on the structure of the *a*-subunit.

The *a*-subunit has been extensively studied by site-directed mutagenesis (for reviews see Refs. 2 and 4). However, there has been no systematic evaluation of the role of proline residues in the *a*-subunit. The unique properties of proline have several implications for protein structure. Incorporation of a proline residue into an α-helix causes a kink and results in unsatisfied backbone hydrogen bonds. More energy will therefore be required to bury a proline residue in the centre of a globular protein or in a transmembrane helix. Proline is rarely found in α-helices within globular proteins, but is found more frequently than expected by chance in transmembrane helices of transporter proteins [5]. This has led to the suggestion that proline residues may have a functional role in transmembrane helices [5,6]. This could be either mechanistic – for example, with *cis*/*trans* isomerization being required for function – or structural. Proline residues have been found to have an active role in transporter gating in the *pst* system of *E. coli* [7].

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A current model of the F_0F_1-ATPase [8] places several proline resides in or near the membrane. Pro-103 is the only proline residue to lie within the membrane, suggesting that it may have a functional role. Two other proline residues, Pro-122 and Pro-143, lie just outside the membrane and are near two residues which are thought to form a charge pair which has a role in maintaining the structure of the *a*-subunit [9]. A mutation in Pro-143 has been found to affect the assembly of the F_0F_1-ATPase [10]. In the present work, the roles of these proline residues were investigated by replacing them with alanine, both singly and in combination with other mutations affecting *a*-subunit stability.

TABLE I

Oligonucleotides used in site-directed mutagenesis experiments. The altered bases are underlined

Oligonucleotide	Amino acid change
5′-CTGATTGCTGCGCTGGC-3′	Pro-103 → Ala
5′-GATTTACTGGCTATCGACCTG-3′	Pr-122 → Ala
5′-CGTGTGGTTGCGTCTGCGGAC-3′	Pro-143 → Ala
5′-TCAATATTAACTCCATGTT-3′	Asp-44 → Asn
5′-TGCCTATCAACCTGCTGAA-3′	Asp-124 → Asn
5′-CCTGCACTGCAAGTGGTTCCG-3′	Arg-140 → Gln
5′-TTTACTGGCTATCAACCTGCTG-3′	Pro-122 → Ala, Asp-124 → Asn
5′-CTGCACTGCAGGTGGTTGCGTCT-GCGG-3′	Arg-140 → Gln, Pro-143 → Ala

Materials and Methods

Enzymes and chemicals

All chemicals and enzymes used were of the highest quality available. Oligonucleotides were synthesized by the Biomolecular Resource Facility, A.N.U., Canberra. [α-^{35}S]dATPαS was obtained from Amersham (Australia) Pty Ltd.

Media and growth of organisms

All bacterial strains were derived from *Escherichia coli* K12. The mineral salts minimal medium used and additions were as described previously [11]. Cells for the preparation of membranes were grown in 14-litre fermenters as described previously [12]. The minimal salts medium in the fermenters was supplemented with 5% (v/v) Luria broth [13].

Turbidities of cultures were measured with a Klett-Summerson colorimeter. Growth yields were measured as turbidities after growth had ceased in medium containing limiting (5 mM) glucose.

Site-directed mutagenesis and construction of plasmids

Procedures for the preparation of phage and plasmid DNA were based on standard techniques [14]. Mutants were obtained as outlined in the Amersham handbook 'Oligonucleotide-directed in vitro mutagenesis system'. The oligonucleotides carrying the appropri-

TABLE II

Properties of mutant strains

Strain	Growth on succinate	Growth yield on 5mM glucose (Klett units)	ATPase activity μmol/min/mg protein)	% fluorescence quenching		
				native		stripped
				NADH dependent	ATP dependent	NADH dependent
coupled control [a]	+	200	0.7	88	87	56
uncoupled control [a]	−	136	0.2	89	0	86
P103A	+	178	0.3	89	56	66
P122A	+	199	0.6	79	89	80
P143A	+	191	0.7	86	83	78
P122A + P143A	+	200	0.6	85	90	72
P122A + D124N	−	146	0.3	76	0	81
P122A + R140Q	+	171	0.3	90	20	92
P143A + D124N	+	174	0.5	84	73	79
P143A + R140Q	+	178	0.6	82	76	84
D124N + R140Q [b]	+	193	0.6	82	30	80
D44N + P122A	−	135	0.4	88	5	88
D44N + P143A	−	160	0.6	86	18	86

[a] The coupled control was formed by transforming strain AN727 with a plasmid generated as described in Materials and Methods except that it carried a wild type *a*-subunit. To generate the uncoupled control the vector pAN174 was transformed into strain AN727.

[b] Ref 9.

ate substitutions are shown in Table I. The presence of each mutation was confirmed by DNA sequencing. DNA sequences were determined by the dideoxy chain-terminating method of Sanger et al. [15] using a Pharmacia T7 dideoxy nucleotide sequencing kit with [α-^{35}S]dATPαS. A 2.2 kb *Hin*dIII-*Cla*I fragment carrying the mutated *uncB*, as well as *uncE* and *uncF*, was subcloned from M13mp18 replicative form into the vector pAN174 as previously described [9]. The correct plasmid was identified as one which conferred on strain AN1440 (*uncF*469) the ability to grow on succinate minimal medium in the presence of chloramphenicol. Restriction analysis confirmed the presence of the desired insert. Plasmids containing the correct insert were then used to transform strain AN727 (*uncB402*). One transformant from each was purified and retained for further work.

Preparation of subcellular fractions

The preparation and treatment of subcellular fractions were as previously described [16].

Other methods

ATPase and atebrin fluorescence quenching activities were assayed as previously described [11]. Protein concentrations were determined using Folin's phenol reagent [17] with bovine serum albumin as standard.

Results

The effect of substituting alanine for Pro-103, Pro-122 or Pro-143

The oligonucleotides shown in Table I were used to generate the mutants P103A, P122A, P143A and P122A + P143A. All three single mutants, as well as the double mutant P122A + P143A, were able to grow on succinate indicating that they were capable of oxidative phosphorylation (Table II). Growth yields for P122A, P143A and P122A + P143A were the same as for the coupled control while the growth yield of P103A was slightly less (Table II). Membranes were prepared from each strain and ATPase and proton pumping activities were determined. P122A, P143A and P122A + P143A were similar to the coupled control except that the NADH quench was retained in membranes from which the F_1-ATPase had been removed by dialysis (Table II). P103A had reduced ATPase activity and reduced ATP-dependent quenching as well as showing partial retention of the NADH quench after dialysis.

The effect of combining the proline mutations with other mutations affecting a-subunit stability

The phenotype of the Pro → Ala mutations described above was similar to that reported for the mutants D44N, D124N and R140Q [9]. This phenotype is indicative of a structural role for the residue mutated

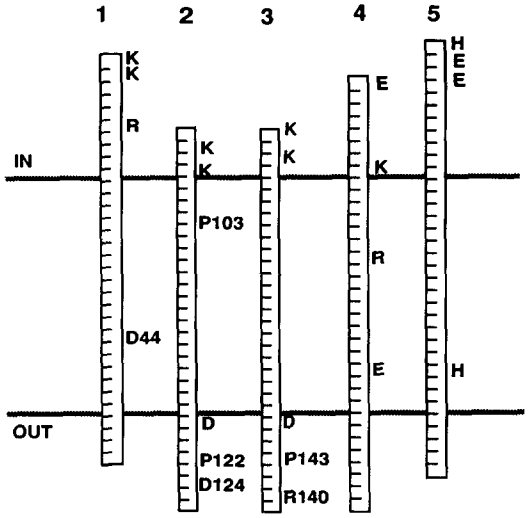

Fig. 1. Proposed transmembrane helices of the *a*-subunit from *E. coli* F_0-ATPase. Charged residues in or near the membrane are shown and those discussed in the text are numbered. D, aspartic acid; E, glutamic acid; H, histidine; K, lysine; P, proline; R, arginine.

because the retention of NADH quenching after removal of the F_1-ATPase suggests an alteration in the structure of the *a*-subunit which prevents passive proton translocation. Asp-124 and Arg-140 are thought to form a charge pair [9] and the proximity of Pro-122 and Pro-143 to these two residues (Fig. 1) makes it likely that these proline residues may be required for the correct positioning of Asp-124 and Arg-140. The fact that the same phenotype is obtained when each of these four residues is mutated singly supports this idea. To test it further, combinations of these mutations were examined. If the effect of the P122A and P143A mutations is due solely to disruption of the charge pair, combining either of these mutations with the D124N or R140Q mutation should result in a strain with a similar phenotype to strains carrying each of the single mutations.

It has been found previously that combining either of the D124N or R140Q mutations with D44N resulted in a complete loss of oxidative phosphorylation, presumably because two such destabilizing mutations could not be tolerated [9]. The P122A and P143A mutations were therefore also combined with D44N to determine whether the same effect was obtained.

Both the double mutants involving Asp-44 (D44N + P122A and D44N + P143A) showed a complete loss of oxidative phosphorylation, as indicated by the inability of these strains to grow on succinate and their growth yields which were similar to that of the uncoupled control (Table II). Membrane preparations from these strains had lowered ATPase activity and minimal ATP-dependent proton pumping (Table II). A similar phenotype was also observed for the mutant P122A + D124N. The other three double mutants, P122 + R140Q, P143A + D124N and P143A + R140Q, had

normal or near normal ATPase activity but retained the NADH quench after dialysis (Table II), as occurred in the strains carrying single mutations. However, in these double mutants the growth yield on glucose was intermediate between the coupled and uncoupled controls and a slight reduction in the ATP-dependent quenching was also evident.

Discussion

Mutations in all three proline residues (Pro-103, Pro-122 and Pro-143) resulted in functional instability of the *a*-subunit. In all three Pro → Ala substitutions, oxidative phosphorylation could still occur but a loss of passive proton translocation through the F_0 after the F_1-ATPase had been removed was observed. This suggested that the correct *a*-subunit structure is not maintained unless the F_1-ATPase is present. Thus, the three proline residues may each have a structural role. A similar result has been obtained previously for Pro-143, where a chromosomal mutation of Pro-143 → Ser was isolated [10]. However, this mutation had more severe effects in that growth on succinate minimal medium was significantly slower than for a normal strain. This may indicate some difficulty with assembly since the presence of the mutant gene on a plasmid, as opposed to a chromosomal location, would result in a higher concentration of the mutant protein. A gene dosage effect has been observed previously with mutations affecting the *c*-subunit [18]. Alternatively, the different effects may be due to the presence of serine rather than alanine at position 143.

Pro-103 is located within the membrane in a current model of the *a*-subunit structure [8] (Fig. 1) and is therefore likely to have an important structural or functional role. We have shown that Pro-103 is not essential for F_0F_1-ATPase activity, but the low ATPase activity in the P103A mutant indicates that this mutation affects assembly of the F_0F_1-ATPase. Pro-103 may have a role in positioning helices such that the charged residues involved in proton translocation are correctly aligned. Such a structural role has been proposed for proline residues in other systems (see Ref. 6).

Pro-122 and Pro-143 are of interest because the model of the *a*-subunit structure shown in Fig. 1 places them next to each other on putative helices 2 and 3. A charge pair between Asp-124 on helix 2 and Arg-140 on helix 3 has been proposed [9]. The proximity of the two proline residues to this charge pair and the fact that they are adjacent raises the possibility that they may have a role in the orientation of helices 2 and 3 such that the charge pair can form. The results obtained are consistent with this. The P122A and P143A mutations resulted in the same phenotype as previously observed for the D124N and R140Q mutations [9]. When both proline residues were substituted by alanine, the effect was no worse than the substitution of either one alone. This would be expected if the loss of either proline alone was sufficient to destroy the charge pair; loss of the second proline would then have no further effect. Furthermore, combining the proline mutations with other mutations causing functional instability of the *a*-subunit also suggests that the main effect of the proline mutations is to prevent formation of the charge pair between Asp-124 and Arg-140.

The P122A and P143A mutations were each combined with both D124N and R140Q. Since all four single mutations are thought to exert their effect by destroying the charge pair, any double mutant should be no worse than the single changes. The D124N and R140Q double mutant has previously been shown to have a similar phenotype to the single mutants [9]. This was also found to be true for three of the four double mutants described in this work (P122A + R140Q, P143A + D124N, P143A + R140Q) although the effect was a little more severe, resulting in a reduced growth yield on glucose. In the fourth double mutant, P122A + D124N, F_0F_1-ATPase activity was completely lost, as indicated by the failure of this mutant to grow on succinate minimal medium. These results do suggest that the main effect of the proline mutations is to prevent the charge pair from forming although the loss of both Pro-122 and Asp-124 appears to have additional effects.

Of particular importance are the results obtained with the four double mutants involving one residue on helix 2 and one on helix 3 (P122A + P143A, P122A + R140Q, P143A + D124N, D124N + R140Q). All four mutants are capable of significant oxidative phosphorylation. In models of *a*-subunit structure other than the one presented here, these residues are not located near each other [19,20]. If this were the case, the effects of these mutations might be expected to be additive since a different location would imply a different role. Such a cumulative effect occurs when the D44N mutation is combined with any one of the P122A, P143A, D124N or R140Q mutations. All four of these double mutants are unable to grow on succinate minimal medium. It therefore appears that two such destabilizing mutations are sufficient to prevent assembly of a normal *a*-subunit. These results provide further support for the role of Pro-122 and Pro-143 in allowing the Asp-124/Arg-140 charge pair to form.

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