A Mutation in Which Alanine 128 Is Replaced by Aspartic Acid Abolishes Dimerization of the b-Subunit of the F0F1-ATPase from Escherichia coli

Susan M. Howitt, Andrew J. W. Rodgers, Peter D. J. effrey, and Graeme B. Cox

From the Division of Biochemistry and Molecular Biology, John Curtin School of Medical Research, Australian National University, GPO Box 334, Canberra City, ACT 2601 Australia

Site-directed mutagenesis was used to investigate the roles of a short series of hydrophobic amino acids in the b-subunit of the Escherichia coli F0F1-ATPase. A mutation affecting one of these, G131D, had been previously characterized and was found to interrupt assembly of the F0F1-ATPase (J ans, D. A., Hatch, L., Fimmel, A. L., Gibson, D., and Cox, G. B. (1985) J. Bacteriol. 162, 420-426). To extend this work, aspartic acid was substituted for each one of the residues from positions 124 to 132. The properties of mutants in this series are consistent with the region from Val124 to Gly131 forming an α-helix. Two of the mutations, V124D and A128D, resulted in a similar phenotype to the G131D mutation. This suggested that Val124, Ala128, and Gly131 form a helical face which may have a role in inter- or intrasubunit interactions. This was tested by overexpressing and purifying the cytoplasmic domains of the wild type and A128D mutant b-subunits. Sedimentation equilibrium centrifugation indicated that the wild type domain formed a dimer whereas the mutant was present as a monomer.

The F0F1-ATPase enzyme complex catalyzes 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 F0 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 F1-ATPase portion of the complex and which retains ATP hydrolytic activity when removed from the membrane.

The a, b, and c subunits are all required for the formation of a proton pore (2) and are present in a stoichiometry of 1:2.6-12 (3). The b-subunit consists of a single transmembrane helix at the N terminus with the rest of the protein extending into the F1-ATPase. The cytoplasmic domain is strongly hydrophilic and is predicted to form two long α-helices separated by a turn (4, 5). Existing evidence is consistent with this structure. The cytoplasmic domain has been expressed and purified and an elongated structure with a high α-helical content was indicated by sedimentation equilibrium centrifugation and circular dichroism spectroscopy (6). The purified cytoplasmic domain of the b-subunit was found to form a dimer which was capable of interacting with the F1 (6). Cryo-electron microscopy of the complex formed between the F1 and the purified dimer indicated that one of the b subunits showed an increased density, suggesting that the b subunit may interact with a specific β subunit (7). Interactions between the hydrophilic domain of the b-subunit and F1 subunits may also be required for assembly of the F0F1-ATPase. A number of b-subunit mutations which affect assembly of the ATPase complex have been isolated (8-11). One of these, G131D, resulted in the b-subunit being able to assemble into the membrane but assembly of the whole complex was blocked at the 1n2p stage, suggesting that this mutation interfered with the b-subunit’s interaction with one of the minor subunits (8).

Although the cytoplasmic domain of the b-subunit is extremely hydrophilic, Gly131 lies in a short stretch of hydrophobic residues (Val124 to Ala132). These lie near the end of a larger region predicted to be α-helical. In the present study the effect of replacing each of these hydrophobic residues with aspartic acid was examined.

EXPERIMENTAL PROCEDURES

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-35S]dATP5 was obtained from Amersham (Australia) Pty Ltd. Glutathione-linked agarose resin and thrombin were obtained from Sigma.

Media and Growth of Organisms—All bacterial strains were derived from E. coli K12. The mineral salts minimal medium used and additions were as described previously (12). Cells for the preparation of membranes and for the overexpression of GST fusion proteins were grown in 14-liter fermenters as described previously (13). Cells to be used for membrane preparation were grown in minimal salts medium supplemented with 5% (v/v) Luria broth (14) while cells expressing GST fusions were grown in Luria broth supplemented with 33 mM glycerol.

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 (15). Mutants were obtained as outlined in the Amersham handbook “Oligonucleotide-directed in vitro mutagenesis system.” The presence of each mutation was confirmed by DNA sequencing. DNA sequences were determined by the dideoxy chain termination method of Sanger et al. (16) using a Pharmacia T7 dideoxy nucleotide sequencing kit with [α-35S]dATP5. A 2.2-kilobase HindIII-Clal fragment carrying the uncB, uncE, and mutated uncF genes was subcloned from M13 mp18 replicative form into the vector pAN174 as described previously (17). The correct plasmid was identified as one which conferred on strain AN943 (uncE429) 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 AN943.

The abbreviations used are: ATP5S, adenosine 5’-triphosphate; GST, glutathione S-transferase; HPLC, high performance liquid chromatography.
AN1440 (uncF 469) (18). The mutant plasmids generated are shown in Table I.

One transformant from each was purified and retained for further work. A similar plasmid carrying the G131D mutation was generated by digesting pAN257 (8) with HindIII and Clal and subcloning the 2.2-kilobase fragment into pAN174 as described above. Coupled and uncoupled controls were produced by transforming strain AN1440 with pAN495, which carries the wild type uncB, uncE, and uncF genes (17), and pAN174, respectively.

The vector used to construct GST fusion proteins was p2GEX-4T, which carries two copies of the gene encoding glutathione S-transferase (20). A BamHI site was introduced into the wild type and A128D mutant subunit genes by site-directed mutagenesis, replacing the codons for Pro-27 and Pro-28. This creates a thrombin cleavage site at the junction between GST and the subunit portion, allowing the release of the subunit protein with digestion with thrombin. A 2.1-kilobase BamHI/EcoRI fragment carrying the portion of the uncF gene encoding the cytoplasmic domain of the subunit and the uncF gene was then subcloned into the BamHI/EcoRI sites of the vector. After confirmation of the presence of the insert by restriction analysis, plasmids were used to transform strain AN3347 which also contains the plasmid pGroESL (Table I).

Preparation of Subcellular Fractions—The preparation and treatment of subcellular fractions were as described previously (21).

Expression of GST Fusion Proteins and Purification of b-subunit Cytoplasmic Domains—Production of the fusion protein was induced by addition of isopropyl-β-D-thiogalactopyranoside to a concentration of 0.1 mM when the cell density had reached 200 Klett units. After a further 2-hour growth, the cells were collected by centrifugation and washed and resuspended in STEM buffer (21). Cells were disrupted in a French press and the cell debris and unbroken cells removed by centrifugation (30 min at 30,000 x g at 4 °C). Since initial experiments showed that a significant amount of the b-subunit cytoplasmic domain/GST fusion proteins was soluble, the supernatant was used directly for affinity chromatography on a glutathione-linked agarose resin. The resin was washed in SMP buffer which contained 86 g/liter sucrose, 20 mM MgSO4, and 10 mM sodium phosphate (pH 7.0). The fusion proteins contain an engineered high affinity thrombin recognition site so the b-subunitcytoplasmic domain was released into SMP buffer by cleavage with thrombin (10 units/ml, 4 h at 25 °C). The eluate was then treated with 1 mM phenylmethylsulfonyl fluoride to inactivate thrombin and the b-subunit cytoplasmic domain purified by HPLC using a TSK G3000SW column.

Ultracentrifugation—Sedimentation equilibrium experiments were performed in a Beckman Optima XL-A analytical ultracentrifuge using 12-mm path length cells with carbon-filled double-sector centerpieces. The cells contained 100 μl of sample (at a concentration of approximately 0.5 mg/ml) dissolved in SMP buffer, and the solvent sector contained 110 μl of the same buffer as a reference. All experiments were carried out at 4 °C to minimize any potential hydrolysis of the sample. Scans at sedimentation equilibrium of absorbance (A) versus radial distance (r) in centimeters from the axis of rotation were collected at 230 nm. The attainment of sedimentation equilibrium was checked by collecting and superimposing scans at 2-hour intervals. This revealed that equilibrium had been reached in all cases after 6 h at the chosen speed (15,000 rpm). Scans at 360 nm were collected and subtracted from the equilibrium scans to correct for anomalies arising from cell windows. The resulting A versus r data was analyzed using the standard programs supplied with the instrument, XLA EQ and XLAMW, employing the sedimentation equilibrium equation,

$$\langle M_w \rangle = \frac{2RT}{(1 - v\rho_w)} \frac{d\ln(A)}{dr^2},$$

(Eq. 1)

where R is the gas constant, T is the absolute temperature, ω is the angular rotation in radians per second, v is the partial specific volume of the protein, 0.73 in this case, and r is the density of the buffer. The density of SMP buffer, which is significantly higher than that of water due to the presence of the sucrose, was determined using a digital precision densitometer to be 1.048 g/ml. The procedure followed in analyzing the data was to obtain the best fit molecular weight for the sample using the program XLA EQ which assumes the sample is homogeneous and thermodynamically ideal and evaluates the molecular weight corresponding to the best fit straight line through the A versus r data from Equation 1. Point average molecular weights as a function of concentration were also evaluated from Equation 1 using the program XLAMW which fits groups of points along the A versus r scan and uses the slope of the In A versus r2 data at the central point of the group to give a point average molecular weight at the corresponding concentration. Examination of the plot of molecular weight versus concentration indicated whether there was size heterogeneity in the sample. Dependence of weight average molecular weight on concentration was interpreted in terms of the known subunit molecular weight of the protein as described in the text.

Other Methods—ATPase and aterbin fluorescence quenching activities were assayed as described previously (14, 22). Protein concentrations were determined using Folin’s phenol reagent (23) with bovine serum albumin as standard.

RESULTS

Identification and Functional Analysis of a Conserved Hydrophobic Region of the b-Subunit—Hydropathy plots of the b-subunits from several species show two hydrophobic regions (Fig. 1). One of these, at the N terminus of the protein includes 33 amino acids and is generally believed to form a transmembrane helix. The other region includes nine residues toward the C terminus and is more highly conserved than the b-subunit as a whole (Fig. 2A). The high conservation of this region, and its presence in organisms in which two identical b-subunits are incorporated into the ATP synthase, raises the possibility that it may have a functional role in the interaction between the two b-subunits. One residue in this region, Gly131, had previously been replaced with aspartic acid in a strain generated by random mutagenesis and the properties of this mutant suggested that interaction of the b-subunit with one of the minor subunits was affected (8).

To further investigate the role of this region, a series of mutants was constructed in which aspartic acid replaced each

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Table I

| Plasmid     | Genotype  | Codon change in unB gene | Amino acid change encoded | Ref. |
|-------------|-----------|--------------------------|--------------------------|------|
| pAN1147     | G131D     | N/A                      | N/A                      | 17   |
| pAN1157     | Fusion of uncF from codon 29 to the gene for GST, Ap' | A128D Fusion of uncF from codon 29 to the gene for GST, Ap' | This study |

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AN1440 (uncF 469) (18). The mutant plasmids generated are shown in Table I. One transformant from each was purified and retained for further work. A similar plasmid carrying the G131D mutation was generated by digesting pAN257 (8) with HindIII and Clal and subcloning the 2.2-kilobase fragment into pAN174 as described above. Coupled and uncoupled controls were produced by transforming strain AN1440 with pAN495, which carries the wild type uncB, uncE, and uncF genes (17), and pAN174, respectively.

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FIG. 1. Hydropathy plots of b-subunits from different species. Hydropathy plots were calculated using the algorithm of Kyte and Doolittle (24) and a window size of 9 amino acids. The hydrophobic region discussed in the text is underlined. Sequences used were from: E. coli (1), Vibrio alginolyticus (25), Bacillus megaterium (26), and the thermophile, Bacillus PS3 (27).

residue from Val \textsuperscript{124} to Ala \textsuperscript{132}. Each mutant plasmid was transformed into strain AN1440 (uncF 469) which carries the chromosomal mutation Trp\textsuperscript{189} → stop (18). Membranes were prepared from each strain and the growth properties of the mutant strains and the ATPase activity and proton pumping activities of the membranes were determined. The results are shown in Fig. 2B and examination of all the properties of the mutant strains suggests a helical periodicity for this region. The most severely affected strains were those carrying the V124D and A128D mutations. These two strains were unable to grow on succinate and had growth yields typical of the uncoupled control. The membranes had no ATPase activity or ATP-dependent fluorescence quenching activity. The NADH-dependent quenching activity was reduced, indicating that the membranes were permeable to protons. If this region was helical, Val\textsuperscript{124} and Ala\textsuperscript{128} would be one helical turn apart and would lie on the same face of the helix as Gly\textsuperscript{131}. The G131D mutation resulted in similar properties to V124D and A128D although the effects were slightly less severe.

On the opposite side of the helix are Ile\textsuperscript{126} and Ala\textsuperscript{130} and mutation of either of these residues resulted in strains which were similar to the wild type. The introduction of an aspartic acid residue at these positions did have some effect as both the I126D and A130D strains had reduced ATPase activity and reduced levels of ATP-dependent fluorescence quenching. However, both strains showed significant growth on succinate and had growth yields which approached the wild type level. The NADH-dependent fluorescence quenching activity of these two strains was similar to the wild type.

Of the remaining mutants, all except the A132D mutant were unable to grow on succinate. Replacement of Ala\textsuperscript{125}, Leu\textsuperscript{127}, or Val\textsuperscript{129} with aspartic acid was not as deleterious as at positions 124 or 128 because some ATPase function was retained in each of these mutants. The L127D and V129D mutants had reduced NADH-dependent fluorescence quenching activities indicative of proton permeable membranes. The only exception to the apparent helical periodicity of this region was the A132D mutant which had growth properties identical to the coupled control. Residue 132 is the last hydrophobic residue in this stretch. The A128D mutation was selected for further experimentation to investigate the possible role of this region in interaction between the two b-subunits.

Expression and Purification of the Cytoplasmic Domains of the Wild Type and A128D Mutant b-Subunits—Two plasmids which carried the cytoplasmic domains of the wild type and A128D mutant b-subunits fused to GST were constructed as described under “Experimental Procedures.” The plasmids carrying equivalent constructs for the wild type and cytoplasmic domains were introduced into strain AN3347 which also contains a plasmid encoding the two chaperonin proteins GroES and GroEL (19). After induction with isopropyl-\beta-thiogalactopyranoside, it was found that high levels of both fusion proteins were produced and a significant proportion was soluble (Fig. 3). Both the wild type and the A128D mutant proteins were purified by affinity chromatography on a glutathione-linked agarose resin and the b-subunit domains liberated by digestion with thrombin (Fig. 3). The proteins were then further purified by size exclusion HPLC. The wild type protein eluted with an apparent molecular mass of about 160 kDa whereas the A128D mutant protein eluted with an apparent molecular mass of about 85 kDa. The b-subunit domains are 131 amino acids long with glycine and serine as the N-terminal residues preceding leucine (residue 29 of the full-length b-subunit). The predicted molecular mass is 14.2 kDa.

Molecular Mass Determination by Analytical Ultracentrifugation—Analytical ultracentrifugation was used to investigate the self-association properties of the wild type and mutant b-subunit cytoplasmic domains. The absorbance at 230 nm versus radial distance data collected at sedimentation equilibrium were fitted assuming the protein samples were homogeneous in molecular weight and thermodynamically ideal using the program XLAEQ supplied with the Beckman Optima XL-A analytical ultracentrifuge. The latter assumption is valid as the proteins are of low molecular weight and charge in aqueous buffer and of a concentration below 1 mg/ml. The molecular weight obtained for the wild type sample was 28,000 with an estimated error of ±1000. This indicates that the wild type b-subunit cytoplasmic domain forms a dimer, as has previously been reported (6). It was possible to fit all of the data with a single molecular weight of this value although there was slight deviation in the last few high concentration points indicating possible minor aggregation at higher concentration. The mutant protein fitted in the same way gave a best fit using a value of 15,000 ± 500. A constrained fit using a value of 14,000 for the molecular weight also gave an acceptable fit. Thus, the mutant protein is present as a monomer. Deviation of the fitted curve from the experimental points at the highest concentrations in the latter case indicated that the value of 15,000 reflects the presence of minor amounts of higher molecular weight material, possibly some dimer.

DISCUSSION

We have shown that a single mutation, A128D, is sufficient to prevent dimerization of the cytoplasmic domain of the b-
wild type during purification. The A128D mutant b-subunit behaved similarly to the lane 7 material in supernatant following centrifugation to remove beads. The A128D mutant b-subunit is unable to form a dimer suggests that this interaction occurs between the two b-subunits. An earlier study of the G131D mutation (8) reported results consistent with those described here. However, data from two-dimensional gels indicated that assembly of the F₁-ATPase was blocked at the 1₂₁₂ stage (8), implying that one of the minor subunits was unable to interact with the mutant b-subunit. The observation that the membranes prepared from the A128D mutant were permeable to protons implies that the hydrophobic domains of the mutant b-subunits were correctly located in the F₀. The inability of the hydrophilic domains to dimerize may therefore provide a rational explanation for the failure of the mutant to complete F₁ assembly.

A

| Source       | % Identity of b-subunit sequences (cf. E. coli) | Sequence of hydrophobic region |
|--------------|-----------------------------------------------|--------------------------------|
| V. alginolyticus | 72                                      | V₁₂₄ A T L A V A G A          |
| B. megaterium  | 30                                      | V₁₃₆ A S L S V M I A S        |
| PS3           | 31                                      | V₁₃₉ A S L S V L I A S        |
| E. coli       | 100                                     | V₁₂₄ A I L A V A G A          |

B

| Residue replaced by Asp | V₁₂₄ A₁₂₅ I₁₂₆ L₁₂₇ A₁₂₈ V₁₂₉ A₁₃₀ G₁₃₁ A₁₃₂ |
|-------------------------|-----------------------------------------------|
| Succinate growth        | 0 66 20 16 66 4 100                           |
| Growth yield (%)        | 0 39 73 46 0 23 31 ND                         |
| ATPase activity (%)     | 0 85 65 61 65 85 66 ND                       |
| Atebrin quench          | 60 81 81 65 61 65 85 66 ND                   |
| -NADH-dependent         | <5 24 46 <5 <5 <5 36 <5 ND                   |
| -ATP-dependent          |                                               |

Fig. 2. Alignment of the conserved hydrophobic regions and properties of mutants of E. coli in which these residues were replaced by aspartic acid. A, the percentage homology of between the E. coli b-subunit and each of the others was calculated using BestFit in the GCG Sequence Analysis Software Package. Sources of the sequences were as for Fig. 1. Conserved amino acids are in bold. B, properties of mutants of E. coli in which each residue in this region was replaced by aspartic acid. The ability of each strain to grow on solid succinate medium is indicated as + or -.. The values for the growth yield on 5 mM glucose and ATPase activity of each mutant are expressed as percentages, with the values for the coupled and uncoupled controls being set at 100% and 0%, respectively. The ATPase activity of the coupled control was 0.8 μmol/min/mg protein. Atebrin fluorescence quenching activities are given as percentages of the maximum quench. The wild type values were 85% for NADH dependent quenching activity and 92% for ATP-dependent quenching activity.

Fig. 3. SDS-polyacrylamide gel electrophoresis analysis of the purification of the GST/b-subunit (cytoplasmic portion) fusion.

Cells were grown and induced with isopropyl-β-thio-galactopyranoside as described under “Experimental Procedures.” Samples were taken at various stages of the purification procedure and run on a 4–20% polyacrylamide gel which was then stained with Coomassie Blue. Lanes 1 and 8, molecular weight markers; lane 2, cell debris; lane 3, cytoplasmic fraction; lane 4, unbound material following exposure of the cytoplasmic fraction (lane 2) to glutathione-agarose beads; lane 5, material bound to the beads; lane 6, material bound to the beads following digestion with thrombin; lane 7, material in supernatant following centrifugation to remove beads. The A128D mutant b-subunit behaved similarly to the wild type during purification.

subunit. Our results with the wild type b-subunit are consistent with an earlier study (6) which examined the properties of the cytoplasmic domain of the b-subunit. This study found that the soluble portion of the b-subunit could form a dimer which was capable of binding to F₁-ATPase, although with a low affinity. This suggested that it formed a structure similar to the native b-subunits. In both this study and the present one, gel sieve HPLC of the cytoplasmic portion of the wild type b-subunit gave a high apparent molecular mass, consistent with an elongated structure. The native structure was proposed to be a coiled-coil (6), based on a high α-helical content, as indicated by circular dichroism spectroscopy, and on the presence of the characteristic heptad repeat in parts of the b-subunit. Since coiled-coils are stabilized by multiple interhelix contacts, our finding that dimer formation can be prevented by a single mutation would suggest that other structures should be considered.

Ala¹²⁸ lies in the center of a short stretch of hydrophobic amino acids which is conserved among b-subunits from many non-photosynthetic bacteria (Fig. 1). Photosynthetic organisms possess two different homologues of the b-subunit (28, 29) and this region is not present in either one of them. In these organisms it is thought that one of the two b-subunit homologues may replace the pair of identical b-subunits found in non-photosynthetic bacteria. The presence of the conserved hydrophobic region only in species where the F₀F₁-ATPase contains two identical b-subunits is consistent with its role in dimerization. In E. coli, this region is predicted to form part of an extended α-helix (5) and our data suggests that the residues from Ala¹²⁴ to Gly¹³¹ do form a helix. The most severe effects occurred when aspartic acid was placed on the helical face defined by positions 124, 128, and 131. On the opposite face, defined by Ile¹²⁶ and Ala¹³⁰, the mutants showed normal or near normal behavior. Aspartic acid at positions 125, 127, and 129 resulted in intermediate phenotypes, as would be expected if this region does form a helix. The only mutant where results were inconsistent with a helix was the A132D mutant which was similar to the wild type. However, Ala¹³² is the last hydrophobic residue in this stretch and may mark the end of the helix or aspartic acid at this position may interact with neighboring charged residues.

In the normal b-subunit Val¹²⁴, Ala¹²⁸, and Gly¹³¹ may form a helical face which interacts with another hydrophobic surface. In the mutants, this interaction would be disrupted by the introduction of an aspartic acid residue. The finding that the cytoplasmic domain of the A128D mutant b-subunit is unable to form a dimer suggests that this interaction occurs between the two b-subunits. An earlier study of the G131D mutation (8) reported results consistent with those described here. However, data from two-dimensional gels indicated that assembly of the F₁-ATPase was blocked at the 1₂₁₂ stage (8), implying that one of the minor subunits was unable to interact with the mutant b-subunit. The observation that the membranes prepared from the A128D mutant were permeable to protons implies that the hydrophobic domains of the mutant b-subunits were correctly located in the F₀. The inability of the hydrophilic domains to dimerize may therefore provide a rational explanation for the failure of the mutant to complete F₁ assembly.
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Susan M. Howitt, Andrew J. W. Rodgers, Peter D. Jeffrey and Graeme B. Cox

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