Characterization of *Escherichia coli* ATP Synthase β-Subunit Mutations Using a Chromosomal Deletion Strain†

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ABSTRACT: (1) We constructed *Escherichia coli* strain JP17 with a deletion in the ATP synthase β-subunit gene. JP17 is completely deficient in ATP synthase activity and expresses no β-subunit. Expression of normal β-subunit from a plasmid restores haploid levels of ATP synthase in membranes. JP17 was shown to be efficacious for studies of β-subunit mutations. Site-directed mutants were studied directly in JP17. Randomly generated chromosomal mutants were identified by PCR and DNA sequencing, cloned, and expressed in JP17. (2) Eight novel mutations occurring within the putative catalytic nucleotide-binding domain were characterized with respect to their effects on catalysis and structure. The mutations βC137S, βG152D, βG152R, βE161Q, βE161R, and βG251D each impaired catalysis without affecting enzyme assembly or oligomeric structure and are of interest for future studies of catalytic mechanism. The mutations βD301V and βD302V, involving strongly conserved carboxyl residues, caused oligomeric instability of F_1. However, growth characteristics of these mutants suggested that neither carboxyl side chain is critical for catalysis. (3) The mutations βR398C and βR398W rendered ATP synthase resistant to aurovertin, giving strong support to the view that βR398 is a key residue in the aurovertin-binding site. Neither βR398C or βR398W impaired catalysis significantly.

In bacteria and mitochondria, ATP synthesis by oxidative phosphorylation is catalyzed by ATP synthase. ATP synthase is a multisubunit enzyme consisting of two sectors, F_1 and F_o, which are linked by a stalk. It uses the electrochemical transmembrane gradient of H^+ ions to drive ATP synthesis, and the catalytic sites for ATP synthesis are believed to be carried wholly or predominantly on the β-subunits. In bacteria the enzyme may also act in reverse, to hydrolyze ATP and extrude protons [reviewed in Senior (1988, 1990); Futai et al., 1989; Ysern et al., 1988; Fillingame, 1990].

Mutations in the *Escherichia coli* ATP synthase β-subunit that affect catalytic activity but do not disrupt assembly of the enzyme have been shown to be valuable in understanding features of the enzyme's mechanism of action and structure, for example, as recently described in reports by Al-Shawi et al. (1990) and Takeyama et al. (1990). It is to be expected that mutagenesis and reversion analysis methods will provide more insights in the future. Mutations in the β-subunit have previously been obtained by a variety of methods, such as random mutagenesis of chromosomal (Cox & Downie, 1979; Senior et al., 1979; 1983), phage-packaged (Kanazawa et al., 1983), or plasmid-borne DNA (Kironde et al., 1989). Site-directed mutagenesis (Parsonage et al., 1987a) has also been valuable. Mutations carried on plasmids have been expressed previously either in an *E. coli* strain from which all the chromosomal ATP synthase structural genes have been deleted (Takeyama et al., 1990) or in Mu-induced polar mutant strains (Parsonage et al., 1987a). A further possible system for expression of mutations would be to utilize a host strain in which a deletion is created within the chromosomal *uncD* (β-subunit) gene, and this approach is explored in this paper.

Here we constructed *E. coli* strain JP17, which contains a deletion of the central two-thirds of the chromosomal *uncD* (β-subunit) gene, and we demonstrate that normal and mutant β-subunits may be expressed from plasmids in this strain with haploid level of ATP synthase being assembled in the membranes. We also report here the properties of 10 novel mutants. Eight of these mutations are located in the predicted catalytic nucleotide-binding domain in the β-subunit (Senior, 1990) and impair catalysis, either substantially or partially. The other two mutations occur at residue βR398, which we have previously predicted to be part of the aurovertin-binding site (Lee et al., 1989), and were made to further investigate the role of this residue.

Methods

Construction of an E. coli Strain Containing a Deletion within the Chromosomal uncD (β-Subunit) Gene. Plasmid pDP40 was originally constructed by Dr. Derek Parsonage in this laboratory and contains an 8.4-kb *Hind*III–*Bgl*II fragment of *E. coli* chromosomal DNA [encoding the distal part of the *uncG* gene, the whole of *uncD* and *uncC*, and a further region downstream from *uncC*; see Walker et al. (1984)] cloned in vector pUC119 between the *Hind*III and *Bam*HI sites. pDP40 was digested with *Cvn*I and *Xma*III, the vector fragment was gel-purified and treated with Klenow fragment and dNTPs to produce blunt ends, and the ends were ligated. After transformation of strain TGlrA (Rao et al., 1988), the "deletion plasmid" was obtained by rapid plasmid minipreparation and characterized by restriction enzyme digestion. The results demonstrated that a stretch of DNA equal in size to the predicted *Cvn*I–*Xma*III fragment had been deleted. Theoretically, this plasmid should lack bp +60 to +998 inclusive of the *uncD* (β) gene and should encode a β-subunit protein in which residues 20–332 inclusive (out of 459 total) are missing. The deletion was incorporated into the *E. coli* chromosome by transforming strain C2110 (*polA*, *his*) (Cain & Simoni, 1986) with the deletion plasmid, as described in Rao et al. (1988). The deletion was then transduced into strain AN346 (Rao et al., 1988), and a *recA* derivative, designated JP17, was made by P1 transduction of *recA* from strain MV1193. Strain JP17 is Tet^R and we propagate it on tetra-

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cycline-containing media to avoid contamination. Strain JP17 has the genotype Δ*uncD*, *argH*, *pyrE*, *entA*, *recA*::Tn10.

Site-Directed Mutagenesis of the uncD (β-Subunit) Gene. Specific mutations were introduced into the *uncD* (β-subunit) gene according to the method of Taylor et al. (1985), essentially following the Amersham Site-Directed Mutagenesis Kit Handbook and with modifications as described by Rao et al. (1988). The template for mutagenesis was M13mp18 phage containing a *Hin*dIII–*Kpn*I insert encoding the *uncD* (β-subunit) and *uncC* (ε-subunit) genes from plasmid pDP31 (Parsonage et al., 1987a). Mutagenic oligonucleotides were 20-mers synthesized in-house. The mutant codons and amino acid residues were as follows: (a) codon 153 GGT → CGT, βG152R; (b) codon 162 GAG → CAG, βE161Q; (c) codon 162 GAG → CGG, βE161R; (d) codon 302 GAT → GTA, βD301V; (e) codon 303 GAC → GTA, βD302V; (f) codon 399, CGC → TGC, βR398C; (g) codon 399 CGC → TGG, βR398W.

The presence of the intended mutations was confirmed by DNA sequencing at the mutation site in the M13mp18 clones. A suitable restriction fragment containing the mutation was transferred to plasmid pDP31, and the whole restriction fragment sequence was obtained to ascertain that no other mutations were present. The mutant plasmids were then transformed into strain JP17 for characterization of their effects. During this part of the work, an apparently spontaneous mutation was found at codon 138 of the β-gene in one clone. This was a TGT → TCT change, causing the βC137S mutation. This mutation was separated by restriction digestion, and since it was found to have interesting effects, it was characterized along with the site-directed mutations.

Identification of Mutations in the Chromosomal DNA of Haploid uncD Mutant Strains by PCR and DNA Sequencing. Chromosomal DNA was prepared from 2 mL of cell suspension (grown in LB–glucose medium) by a rapid procedure consisting of SDS lysis of the cell pellet, sequential extraction with phenol, and then phenol/chloroform and ethanol precipitation. The forward primer for amplification of the *uncD* (β-subunit) gene was a 20-mer whose 3′ end corresponded to bp −30 before codon 1; the reverse primer was a 20-mer whose 3′ end corresponded to bp +28 beyond the TAA termination codon. Double-stranded DNA (1.45 kb in size) was amplified, separated on agarose gel, eluted, and used for a further round of asymmetric PCR amplification to produce single-stranded DNA. The ssDNA was sequenced to identify the mutation present. A *Sal*I–*Xma*III restriction fragment was used to transfer the mutations from the PCR dsDNA into pDP31 for subsequent expression in JP17.

Molecular Biology Techniques. DNA sequencing was by the dideoxy chain termination method with use of a series of 20-mer oligonucleotide primers spaced approximately 200 bp apart and covering the entire *uncD* (β-subunit) gene. Other techniques followed standard procedures and our previous work (Parsonage et al., 1987a,b; Rao et al., 1988).

Growth of E. coli Cells. Preparation of Membrane Vesicles. Solid and liquid minimal medium with required supplements plus 30 mM glucose or 30 mM succinate was as described by Cox and Downie (1979). Casamino acids were included in succinate plates. Rich medium was LB plus 30 mM glucose. For membrane vesicle preparations, a 50-mL starter culture in minimal medium plus 30 mM glucose plus 2.5% LB was grown to OD ~0.7 at 37 °C and then inoculated into 1 L of the same medium and grown to OD 1.5 (mid log). Cells were harvested and washed and then converted to membrane vesicles in a French pressure cell as described previously (Senior et al., 1979). Growth yields in limiting (3 mM) glucose liquid medium plus 2.5% LB were done in a 15-mL cell suspension at 37 °C. Ampicillin (50 μg/mL) was present in all media used for experiments with plasmid-containing strains.

Biochemical Techniques. Assays of protein, ATPase activity, pH-gradient formation in membrane vesicles, and immunoblotting with anti-*b*, anti-α, and anti-β rabbit polyclonal antibodies were as previously described (Perlin & Senior, 1985; Rao et al., 1987, 1988). Staining of immunoblots utilized TMB–peroxidase substrate from KPI Laboratories, Gaithersburg, MD. Stripping of membrane vesicles with 1 M KSCN and subsequent reconstitution with normal purified F_1 were done as described by Perlin et al. (1983).

Results

Construction and Characterization of a Strain of E. coli Containing a Deletion within the uncD (β-Subunit) Gene. As described in the Methods section, we constructed strain JP17 carrying a deletion within the chromosomal *uncD* gene. The *uncD* gene in the chromosome of this strain would theoretically encode a β-subunit lacking residues 20–332 (out of 459 total residues). The deleted segment of β encodes almost all of the putative catalytic nucleotide-binding domain, encompassed by residues 140–335 approximately (Senior, 1988, 1990).

Strain JP17 showed no growth on succinate plates and showed growth yield typical of an *unc*⁻ strain in limiting 3 mM glucose liquid medium. Therefore, JP17 is incapable of carrying out oxidative phosphorylation. Membrane vesicles from strain JP17 showed zero ATPase activity and zero ATP-driven pH-gradient formation. NADH-driven pH-gradient formation was normal. Whole-cell lysates or membrane vesicles from strain JP17 showed no reactive protein when subjected to immunoblotting with anti-β polyclonal antibody. PCR amplification of chromosomal DNA from strain JP17 using primers that specifically amplify the *uncD* (β-subunit) gene (see Methods) produced a 0.5-kb fragment, in contrast to the 1.45-kb fragment obtained from a normal strain. The PCR fragment from JP17 could not be cut with restriction endonuclease *Sst*I, in contrast to the PCR fragment from the normal strain, as would be predicted. It was, however, digested by *Pvu*II and *Dra*III, as expected. This confirmed the presence of the expected 939-bp deletion in the chromosome.

A plasmid containing only the β-gene (obtained by cloning the PCR β-gene from a normal strain into plasmid pUC119) complemented strain JP17 in growth tests, whereas plasmids expressing ATP synthase genes other than β did not complement strain JP17 to any extent in growth tests. Plasmid pDP31 (Parsonage et al., 1987a), which expresses the β- and ε-subunits of ATP synthase, complemented strain JP17 in growth tests, restoring growth to levels seen in the typical haploid wild-type control strain AN1339 (Perlin et al., 1985). Strain pDP31/JP17 showed ATPase activity in membrane vesicles similar to haploid normal strain AN1339 (approximately 2 μmol of ATP hydrolyzed min^{-1} mg^{-1} at 30 °C pH 8.5) and showed normal NADH- and ATP-driven pH-gradient formation.

Expression and Characterization of Site-Directed Mutations in Strain JP17. Several mutations were introduced into the *uncD* (β-subunit) gene in plasmid pDP31 by site-directed mutagenesis and then expressed in JP17. One aim of this study was to evaluate JP17 as a system for expression and study of β-subunit mutations. However, each of the mutations was also chosen with certain considerations in mind, as follows. (1) Catalysis in ATP synthase could involve a general acid–base catalytic residue. Such a residue might be involved in both unisite and multisite catalysis, or it might be involved in the

Table I: Properties of Site-Directed β-Subunit Mutations Expressed in JP17

mutation	growth on succinate plates	growth yield in limiting glucose (OD_{590})	membrane ATPase activity (% of normal)	pH-gradient formation in membrane vesicles (% quench of acridine orange fluorescence)	
				NADH-induced	ATP-induced
β⁻ (pUC118/JP17)[a]	−	0.53	<1.0	90	0
β⁺ (pDP31/JP17)	+++	1.04	100[b]	90	92
βC137S	+	0.70	3.0	90	11
βG152R	−	0.53	<1.0	92	0
βE161Q	+	0.78	8.5	90	15
βE161R	−	0.53	<1.0	91	0
βD301V	+	0.82	<1.0	73	0
βD302V	+	0.78	<1.0	73	0
βR398C	+++	1.04	88	88	85
βR398W	+++	1.06	100	91	89

[a] Strain pUC118/JP17 was used here and subsequently (Table II) as the negative control to allow use of the same growth medium containing ampicillin. [b] Activity was ~2 μmol min^{-1} mg^{-1} at 30 °C, pH 8.5. A normal control was run alongside each preparation of mutant membranes.

acceleration of the unisite to multisite rate that occurs on nucleotide binding to the second and/or third catalytic sites by being propelled into the catalytic site region by a conformation change. A carboxyl group is a possible candidate. From hypothetical modeling of the β-subunit nucleotide-binding domain (Duncan et al., 1986) and from taking into account very strong cross-species sequence conservation, it appeared that residues βE161, βD301, or βD302 may be candidates. They were accordingly chosen for mutagenesis. (2) Residue βG152, which is strongly conserved in β-subunit of different species, is part of the "homology A" or "glycine-rich loop" region (Walker et al., 1982; Fry et al., 1986) found in numerous, diverse, nucleotide-binding proteins. We obtained one mutation at this residue by random mutagenesis (βG152D, see later), and so the βG152R mutation was introduced to further define the role of this residue. (3) As noted in the Methods section, the mutation βC137S was isolated as a spontaneous mutation. We chose to characterize it because it lies very close to the beginning of the predicted nucleotide-binding domain of β-subunit (Duncan et al., 1986) and a previous mutation at this position, namely βC137Y, was shown to be detrimental to function (Kironde et al., 1989).

Table I (lines 1–8) shows the results obtained for the site-directed mutant strains when growth characteristics of cells and biochemical properties of membrane vesicle preparations were studied. The βC137S and βE161Q mutations gave partial impairment of growth on succinate and limiting glucose, partial impairment of membrane ATPase, and partial impairment of ATP-driven proton pumping. DCCD (50 μM) inhibited the ATPase and ATP-driven proton pumping by ~90% in both mutants. The βG152R and βE161R mutations gave substantial impairment of growth, membrane ATPase, and ATP-driven proton pumping. Therefore, in each of these four mutations, either assembly of the ATP synthase or intrinsic catalytic activity of the ATP synthase were affected.

The βD301V and βD302V mutations caused only partial impairment of growth, but membrane ATPase and ATP-induced proton pumping activities were both very low. Also, NADH-induced pH-gradient formation was reduced (73% quench of acridine orange fluorescence), implying that the membranes were somewhat proton-leaky. Addition of DCCD restored the NADH-induced response to around 90% in βD301V and βD302V membrane vesicles. These data imply that the βD301V and βD302V mutant ATP synthases might be unstable, suffering partial release of F_1 subunits from the membranes. Presumably in vivo there is sufficient intact F_1 on the membrane to allow partial growth on succinate and in limiting glucose. In support of this notion, when the βD301V or βD302V mutations were expressed from plasmid pDP34 instead of pDP31, the growth yields in limiting glucose increased to around OD 0.90–0.91, a significant level of membrane ATPase was now seen in membrane vesicles (~1.25% of normal), and the membrane vesicles were very leaky to protons (NADH-induced acridine orange fluorescence quenching was ~6%). DCCD restored full proton impermeability to those membranes. Plasmid pDP34 encodes and overexpresses all of the ATP synthase structural genes (Maggio et al., 1988), yielding a 3–5-fold amplification of enzyme activity in the membranes. Thus, the enhanced membrane ATPase activity would be due to a larger number of F_1 molecules and the enhanced proton leakiness to a larger number of "unplugged" F_o complexes. Therefore, the defect in the βD301V and βD302V mutants is apparently one of lessened oligomeric stability of F_1 subunits in ATP synthase. However, intact βD301V and βD302V ATP synthases in vivo appear to have significant catalytic turnover as indicated by the growth tests.

Table II: ATP-Induced pH-Gradient Formation in KSCN-Extracted, F_1-Reconstituted Membrane Vesicles from Site-Directed Mutant Strains

mutation	ATP-induced quench of acridine orange fluorescence (%)
β⁻(pUC118/JP17)	4
β⁻(AN817, haploid)[a]	5
β⁺(pDP31/JP17)	84 (0)[b]
β⁺(AN1339, haploid)	84 (0)[b]
βC137S	75
βG152R	76
βE161Q	84
βE161R	83
βD301V	76
βD302V	74

[a] Strain AN817 carries the mutation βG214R (Parsonage et al., 1987b) and shows defective assembly of ATP synthase (Senior et al., 1979). A similar result was obtained with several other known assembly-defective mutant strains. [b] Parentheses indicate result obtained with no F_1 added.

In order to assess whether ATP synthase was normally assembled or assembly-defective in the mutants that showed impaired growth and enzyme activities, two further experimental approaches were used. First, membrane vesicles were extracted with 1 M KSCN and then reconstituted with purified normal F_1, and ATP-driven pH-gradient formation was assayed. This assay tests for the presence of normally assembled, functional F_o in the membrane and the removal ("stripping") of F_1 subunits by 1 M KSCN (Perlin et al., 1983). As shown in Table II, strain pDP31/JP17 yielded a high ATP-induced response in this assay (as did normal haploid strain AN1339)

Table III: Properties of Random Chromosomal β-Subunit Mutants

mutation	growth on succinate plates	growth yield in limiting glucose (OD_{590})	membrane ATPase activity (% of normal)	pH-gradient formation in membrane vesicles (% quench of acridine orange fluorescence)		
				NADH	ATP	KSCN-F_1-ATP[a]
β^+ (AN1339)	+++	0.90	100[b]	96	95	86
βG152D (AN1352)	-	0.48	<1.0	96	0	82
βG251D (AN1572)	-	0.50	<1.0	93	0	72
β^- (JP17)	-	0.48	<1.0	92	0	ND[c]

[a] Membrane vesicles were extracted with 1 M KSCN and reconstituted with normal F_1, and then ATP-driven pH-gradient formation was measured. [b] ~2 μmol min^{-1} mg^{-1} at 30 °C, pH 8.5. [c] ND, not determined.

and strains that did not assemble ATP synthase correctly (e.g., AN817) gave a low response. The strain pUC118/JP17 also gave a low response, showing that the absence of expression of normal β-subunit prevented normal F_0 from occurring in the membranes. The data in Table II show that all the mutants described above assemble a normal F_0, which was stripped of F_1 subunits by KSCN and could functionally rebind normal F_1. This suggests that ATP synthase assembly is normal in each of the mutants.

Second, the amounts of α-, β-, and *b*-subunits in membrane vesicles prepared from normal and mutant strains were assessed by immunoblotting. The βC137S, βG152R, βE161Q, and βE161R mutants all had normal amounts of all three subunits (data not shown). Taken together with the results of Tables I and II, this showed that these four mutants are not assembly-defective and that the assembled ATP synthase is structurally stable and has partial (βC137S and βE161Q) or very low (βG152R, βE161R) catalytic turnover of both ATP synthesis and hydrolysis. The membranes from the βD301V and βD302V mutants had normal amounts of *b*-subunit but lower than normal amounts of α- and β-subunits. This suggests, consistent with the results of Tables I and II and the text above, that ATP synthase assembles in the cell membranes of these mutants but that α- and β-subunits are partially released from the membranes.

Characterization of a Group of Randomly Generated Chromosomal uncD (β-Subunit) Mutants and Expression of the Mutations in JP17. In previous work (Senior et al., 1979, 1983; Parsonage et al., 1987b), we had characterized 22 *uncD* (β-subunit) mutants that were originally obtained by F. Gibson, G. Cox, L. Hatch, and colleagues in Canberra, Australia by random mutagenesis of haploid *E. coli* cells (Cox & Downie, 1979). The Canberra collection contained a further 16 such mutants, each of which had been shown by reversion and genetic analysis (Cox & Downie, 1979) to contain a point mutation in the *uncD* gene that resulted in impaired growth on succinate medium.

These strains were kindly provided to us and each was subjected to the same biochemical analyses as described above for site-directed mutants. They fell into two broad groups. One group, of 12 strains, showed essentially zero membrane ATPase and low (0–25%) ATP-driven quench of acridine orange fluorescence in KSCN-extracted, F_1-reconstituted membrane vesicles. Immunoblots of membrane vesicles from the strains showed deficiency of α and β subunits. These strains were therefore concluded to suffer from defective assembly of ATP synthase, a property that has been noted before in haploid *uncD* (β-subunit) strains (Senior et al., 1983; Noumi et al., 1986). For this reason, this group of strains was not further investigated. A second group, of four strains, showed high (>70%) ATP-driven quench of acridine orange fluorescence in KSCN-extracted, F_1-reconstituted membrane vesicles, implying that ATP synthase assembly was normal. Further investigation of these strains was undertaken, as follows.

Chromosomal DNA was prepared from each strain and the *uncD* (β-subunit) gene was amplified by PCR and sequenced. Strain AN1064 was found to carry the βS174F mutation, and strain AN1417 was found to carry the mutation βM209I. Each of these mutations has been found previously, and each has been well characterized (Parsonage et al., 1987b), so these two strains were not further studied. Two new mutations were identified as follows: (1) in strain AN1352, codon 153 GGT → GAT, residue βG152 → D, and (2) in strain AN1572, codon 252 GGT → GAT, residue βG251 → D. Only the single mutation was present in the β-subunit in each strain.

Table III describes growth characteristics and biochemical properties of these two new mutant strains, as compared to strain AN1339, a normal haploid strain. The data show that ATP synthase has very low catalytic turnover in the mutants. Immunoblotting experiments using anti-α, anti-β, and anti-*b* antibodies were carried out on membrane vesicles obtained from strains AN1339, AN1352, and AN1572, following procedures described above. The results indicated that α-, β-, and *b*-subunits were present in normal ratio and in normal amounts in the mutants (data not shown). Therefore, ATP synthase assembly is not significantly impaired by these mutations.

Starting with the DNA obtained by PCR amplification, the βG152D and βG251D mutations were cloned into plasmid pDP31 (as described in the Methods section) and expressed in strain JP17. Growth characteristics and biochemical characteristics of the resultant strains (pD1352/JP17 and pD1572/JP17) were the same as in the parent haploid strains AN1352 and AN1572.

Site-Directed Mutagenesis of Residue βR398 in the Aurovertin-Binding Site. Aurovertin is an antibiotic that binds to β-subunits of ATP synthase and potently inhibits both ATP synthesis and hydrolysis. It has proven to be a valuable fluorescent probe of conformational events in ATP synthase [e.g., Chang and Penefsky (1974) and Wise et al. (1981)] and appears to report conformational changes close to or within the β-subunit catalytic site that are involved in energy coupling and in catalytic site cooperativity. Residue βR398 was suggested to be part of the binding site for aurovertin, on the basis of the evidence that the mutation βR398H apparently prevented aurovertin binding and rendered ATPase and ATP synthesis activities resistant to the antibiotic (Lee et al., 1989). Also, in bacterial species where the residue equivalent to *E. coli* βR398 is phenylalanine, ATP synthase appears to be aurovertin-resistant (Lee et al., 1989; Hicks & Krulwich, 1990). Here, we made the βR398C and βR398W mutations by site-directed mutagenesis, to further examine the aurovertin-binding site properties.

Growth characteristics and biochemical properties of the βR398C and βR398W mutations expressed in strain JP17 are shown in Table I (lines 9 and 10) and demonstrate that the mutations caused little or no effects on ATP synthase activities. Both mutants had normal amounts of α- and β-subunits in

Table IV: Effects of Aurovertin on the Membrane ATPase Activity of βR398C and βR398W Mutants

mutation	membrane ATPase activity[a] (μmol min^{-1} mg^{-1}) no aurovertin	10 μM aurovertin B	10 μM aurovertin D
β^+ (R398)	2.12	0.15	0.15
βR398C	1.82	1.68	1.76
βR398W	2.12	2.18	2.20

[a] Membrane ATPase activity was assayed at 30 °C, pH 8.5 (see Methods). Aurovertin B (Sigma Chemical Co.) or aurovertin D (kindly donated by Dr. R. B. Beechey and Dr. S. D. Dunn) was added as ethanolic solution so that the final ethanol concentration was ≤ 0.4% (v/v).

their membranes. Table IV shows the sensitivity of the membrane ATPase activity to aurovertin, and it is clear that substitution of residue βR398 by C or W substantially reduces the degree of inhibition.

Discussion

We made a strain of *E. coli*, named JP17, which contains a deletion of the central two-thirds of the chromosomal *uncD* (ATP synthase β-subunit) gene. The deletion removes almost all of the predicted catalytic nucleotide-binding domain and results in zero expression of β-subunit in whole-cell lysates or membranes. Expression of normal β-subunit from plasmid pDP31 in JP17 results in restoration of normal growth characteristics with haploid levels of ATP synthase being incorporated into the cell membrane. It was shown that β-subunit mutations, obtained either by site-directed mutagenesis or cloned from PCR-amplified chromosomal DNA of randomly generated mutant strains, could be expressed and studied with facility in strain JP17. Therefore, this system is useful for study of β-subunit mutations and, potentially, for study of intragenic revertants in β-subunit.

Ten novel β-subunit mutations are described here. Four involved mutation of conserved carboxyl residues. The βE161Q and βE161R mutations did not impair assembly or structural stability of ATP synthase but did inhibit catalytic turnover. Residue βE161 therefore seems to play an important role in the catalytic nucleotide-binding domain, and the mutations are interesting for future kinetic studies, although at this stage it seems that residue βE161 does not have a critical catalytic role as a proton donor/acceptor since the βE161Q mutation retained 8.5% of normal membrane ATPase activity. The βD301V and βD302V mutations each caused structural instability of ATP synthase. The in vivo growth characteristics (which were similar to those of the βE161Q mutant) suggested that there was significant catalysis in vivo in each case. However, study of these mutants would be difficult in light of the apparent structural instability.

Position βG152, part of the glycine-rich loop, is occupied by glycine in ATP synthase β-subunits of all species sequenced to date. The mutations βG152D and βG152R were found to inhibit catalytic turnover very strongly. Despite the substitutions of a large, positively or negatively charged residue for glycine, ATP synthase assembly and structure appeared normal in each case and the two mutants are therefore important for future work on the role of this strongly conserved region in catalysis.

We previously noted that residue βC137 lies close to the beginning of the predicted catalytic nucleotide-binding domain and that the mutation βC137Y impaired catalytic turnover (Kironde et al., 1989). Here we found a second mutation at this position, βC137S, which reduced membrane ATPase to 3% of normal. This was a surprisingly large effect, given that the substitution is C → S. By comparison, when plasmid pAN216 carrying the βC137Y mutation (Kironde et al., 1989) was expressed in JP17, the resultant membrane ATPase activity was 11.3% of normal, substantially higher than that with the βC137S mutant.

The βG251D mutation was also found to strongly inhibit catalysis, and it did not impair assembly or oligomeric stability of ATP synthase. Residue βG251 is completely conserved in β-subunits from different species and lies 10 residues away from the "homology B" region (Walker et al., 1982) in the predicted catalytic nucleotide-binding domain of β-subunit.

It is noteworthy that each of the mutations discussed so far lies within (or very close to) the putative catalytic nucleotide-binding domain of β-subunit. It is interesting in this regard to make a summary of the locations of mutations found to date. Of the approximately 30 different reported single point mutations in β-subunit that allow assembly of an ATP synthase with normal oligomeric structure but with impaired catalytic turnover, all occur within or very close to the predicted catalytic nucleotide-binding domain [i.e., between residues 137 and 335; see Senior (1990) for review]. Fourteen of the total were obtained by random mutagenesis.

The final two mutations reported in this paper involved residue βR398 in the predicted aurovertin-binding site. As described above, aurovertin has proven to be a useful reporter probe of energy-linked and nucleotide-induced conformational changes in ATP synthase β-subunit. However, it has disadvantages, namely, that it binds noncovalently and is a potent inhibitor of ATP synthesis and hydrolysis. We made the substitutions βR398C and βR398W and showed that neither mutation significantly affected ATP synthesis or ATPase activity, although both rendered the ATPase activity resistant to aurovertin. Thus, the accumulated data now show that while the residues R, H, F, C, and W all support full or nearly normal catalytic activity when present at position 398, only R facilitates aurovertin-binding and inhibition. In the future, by attaching covalently linked fluorescent probes to the substituted cysteine in the βR398C mutant or by following intrinsic fluorescence of βR398W, we hope to obtain information regarding conformational changes that occur in ATP synthase during ATP synthesis and hydrolysis.

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Registry No. C, 52-90-4; G, 56-40-6; E, 56-86-0; D, 56-84-8; R, 74-79-3; ATP synthase, 37205-63-3; aurovertin, 11002-90-7; hydrogen ion, 12408-02-5.

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Dependence of Kinetic Parameters of Chloroplast ATP Synthase on External pH, Internal pH, and ΔpH

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ABSTRACT: ATP synthesis by the membrane-bound chloroplast ATPase in the oxidized state of its γ disulfide bridge was studied as a function of the ADP concentration, ΔpH, and external pH values, under conditions where ΔpH was clamped and delocalized. At a given pH, the rate of phosphorylation at saturating ADP concentration (V_{max}) and the Michaelis constant K_m (ADP) depend strictly on ΔpH, irrespective of the way the ΔpH is generated: there evidently is no specific interaction between the redox carriers and the ATPase. It was also shown that both K_m (ADP) and V_{max} depend on ΔpH, not on the external or internal pH. This suggests that internal proton binding and external proton release are concerted, so that net proton translocation is an elementary step of the phosphorylation process. These results appear to be consistent with a modified "proton substrate" model, provided the ΔG_0 of the condensation reaction within the catalytic site is low. At least one additional assumption, such as a shift in the pK of bound phosphate or the existence of an additional group transferring protons from or to reactants, is nevertheless required to account for the strict ΔpH dependence of the rate of ATP synthesis. A purely "conformational" model, chemically less explicit, only requires constraints on the pK's of the groups involved in proton translocation.

In chloroplasts, the electrochemical proton gradient, generated by an electron transfer chain, gives the energy required for ATP synthesis (Mitchell, 1961). This energy is stored in phosphate bonds within the F_0F_1 ATP synthase, which consists of a transmembraneous part, the proton channel F_0, and an extrinsic part, F_1, responsible of the catalytic activity.

The detailed mechanism of ATP synthesis, as well as the different roles of the proton gradient, are not yet elucidated. With regard to the energy-coupling events during ATP synthesis, two main models have been described. On the one hand, the proton is considered as a true substrate, which directly interacts with P_i, allowing ATP formation, so its energy is

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