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H^+-ATPase of *Escherichia coli*

AN *uncE* MUTATION IMPAIRING COUPLING BETWEEN F_1 AND F_o BUT NOT F_o-MEDIATED H^+ TRANSLOCATION*

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The *uncE114* mutation from *Escherichia coli* strain K_{11} (Nieuwenhuis, F. J. R. M., Kanner, B. I., Gutnick, D. L., Postma, P. W., and Van Dam, K. (1973) *Biochim. Biophys. Acta* 325, 62–71) was characterized after transfer to a new genetic background. A defective H^+-ATPase complex is formed in strains carrying the mutation. Based upon the genetic complementation pattern of other *unc* mutants by a λ*uncE114* transducing phage, and complementation of *uncE114* recipients by an *uncE*$^+$ plasmid (pCP35), the mutation was concluded to lie in the *uncE* gene. The *uncE* gene codes for the ω subunit ("dicyclohexylcarbodiimide binding protein") of the H^+-ATPase complex. The mutation was defined by sequencing the mutant gene. The G → C transversion found results in a substitution of Glu for Gln at position 42 of the ω subunit in the F_o sector of the H^+-ATPase. The substitution did not significantly impair H^+ translocation by F_o or affect inhibition of H^+ translocation by dicyclohexylcarbodiimide. Wild-type F_1 was bound by *uncE114* F_o with near normal affinity, but the functional coupling between F_1 and F_o was disrupted. The uncoupling was indicated by an H^+-leaky membrane, even when saturating levels of wild-type F_1 were bound. Disassociation of F_1 from F_o under conditions of assay did partially contribute to the H^+ leakiness, but the major contributor to the high H^+ conductance was F_o with bound F_1. The F_1 bound to *uncE114* membranes exhibited normal ATPase activity, but ATP hydrolysis was uncoupled from H^+ translocation and was resistant to inhibition by dicyclohexylcarbodiimide. The F_1 isolated from the *uncE114* mutant was modified with partial loss of coupling function. However, this modification did not account for the uncoupled properties of the mutant F_o described above, since these properties were retained after reconstitution of mutant membrane (F_o) with wild-type F_1.

A reversible, H^+-translocating ATPase in the inner membrane of *Escherichia coli* couples the synthesis of ATP with H^+ translocation through the complex during oxidative phosphorylation. The enzyme is similar to that found in mitochondria, chloroplasts, and a variety of other bacteria (1–3). It is made up of two structurally and functionally separable sectors termed F_1[1] and F_o. The extrinsic F_1 sector is the ATPase moiety of the complex, and F_o is the transmembrane, H^+-translocating element. The *E. coli* F_1F_o complex is composed of eight subunits that are coded for by the genes of the *unc* operon (1, 3–6). A number of *unc* mutants have been described, and their chemical characterizations have provided important insight into the mechanism and function of subunits (1, 3, 5–8). We report here on a novel type of mutant in which ATP hydrolysis in the F_1 sector is uncoupled from H^+ translocation by F_o. The point mutation causing the defect lies in the *uncE* gene and results in an alteration of ω, one of the three subunits of F_o. ω is a small hydrophobic protein that is extracted from F_o and is soluble in chloroform/methanol.[2] A variety of experiments indicate that the ω subunit plays a direct role in H^+ translocation (7–9). Reaction of DCCD with an aspartyl (glutamyl) residue of the protein specifically blocks H^+ translocation and ATP hydrolysis or synthesis by a properly coupled F_1. The equivalent of the ω protein has now been purified and sequenced from 10 species (8). The most conserved region of the protein is a polar region lying between two long hydrophobic stretches of amino acids, both of which are widely presumed to span the lipid bilayer (1, 3, 7–9). ω is presumed to fold in the membrane like a hairpin with the central polar region extending from the membrane as a loop. Within the polar loop region is a sequence of three conserved amino acids, Arg-(Gln or Asn)-Pro. In the mutant characterized here, the Gln in this sequence is substituted by Glu and the substitution results in an uncoupling of F_1 from F_o.

The mutant strain K_{11} was initially described by Nieuwenhuis *et al.* (10) in 1973. A decade later, the mutant allele from strain K_{11} was defined by genetic complementation and designated *uncE114* (11). Based upon the original reports (10, 12), the K_{11} mutant appeared to be unique in comparison to other F_o-type mutants (see Ref. 3 for discussion). Mutant membranes retained F_1-ATPase, but the bound ATPase was not inhibited by DCCD. In contrast to other F_o-type mutants, membranes from mutant K_{11} appeared to be functional in F_o-mediated H^+ translocation as judged indirectly by the extent

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[1] The abbreviations used are: F_1, extrinsic ATPase sector of H^+-ATPase complex; F_o, intrinsic H^+-translocating sector of H^+-ATPase complex; H^+-ATPase, H^+-translocating ATPase complex composed of F_1 and F_o; *unc*, genetic locus for H^+-ATPase genes in *E. coli*; ACMA, 9-amino-6-chloro-2-methoxyacridine; CCCP, carbonyl cyanide *m*-chlorophenylhydrazone; DCCD, dicyclohexylcarbodiimide; HEPES, *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid; MOPS, 3-(*N*-morpholino)propanesulfonic acid; SDS, sodium dodecyl sulfate; Tris, tris(hydroxymethyl)aminomethane.

[2] The ω subunit of F_o is commonly referred to as the "DCCD binding protein."

of succinate-driven quenching of ACMA fluorescence. In the study described below, we have confirmed the general features of the original work on mutant strain K_{11} and extended the characterization of phenotype. The point mutation defined here uncouples ATP hydrolysis by F_1 from H^+ translocation by F_o, even though F_1 is bound to F_o with near normal affinity. The substitution does not measurably alter the rate of F_o-mediated H^+ translocation.

EXPERIMENTAL PROCEDURES

Bacterial Strains and Genetic Methods—Strain K_{11} (10) was provided by R. Simoni (Stanford University). Strain MM973 was constructed by P1 transduction from donor strain K_{11} (*uncE114, pro, his, gal, ara, mal, rpsL, thi-1*, $T6^R$) to recipient strain AN346 (*ilvC7, entA403, pyrE41, argH1, rplL109, thi, supE44*) (13) by selection for *ilv*$^+$ transductants that could not grow on succinate minimal medium. Strain MM180 is the isogenic *ilv*$^+$, *unc*$^+$ derivative of strain AN346 (11). Strain MM994 (*uncE114*) and MM833 (*unc*$^+$) are *recA* derivatives of strains MM973 and MM180, respectively, constructed as described (11). A λ*uncE114* transducing phage was generated as described (11). Diploid strain MM1097 (λ*uncE114*/λ*cI857S7*/*uncE114, bglR, asnA31, asnB32, thi-1*) was used as the source of λ transducing phage for complementation analysis or for DNA sequencing. Strain MM1208 is a derivative of strain HB101 (14) transformed with plasmid pMM114, a plasmid carrying the *uncE114* gene. Plasmid pCP35 (*uncE*$^+$) has been described (15). The methods used for P1 transduction, λ transduction, λ*unc* complementation analysis, pCP35 complementation analysis, and determination of growth yield have been described (11, 15).

Growth of Cells for Biochemical Analysis—Strains MM180 or MM973 were grown at 37 °C to late exponential phase in M63 minimal medium (16) supplemented with 0.7% (w/v) glucose, 5 μg/ml of thiamine hydrochloride, 1 mM L-arginine, 0.5 mM uracil, and 40 μM 2,3-dihydroxybenzoic acid (using 1 liter of medium/4-liter flask and shaking at 200 rpm). Alternatively, 10-liter batches of cells were grown in a New Brunswick fermentor in minimal medium containing 0.1 M potassium phosphate, pH 7.5, 0.4 mM Na_2SO_4, 47 mM NH_4Cl, 0.8 mM $MgCl_2$, 1.8 μM $FeSO_4$, supplemented with 1.4% (w/v) glucose, 5 μg/ml of thiamine hydrochloride, 5 mM L-arginine, 2.5 mM uracil, and 100 μM 2,3-dihydroxybenzoic acid. When cells in 10-liter batches reached a density of approximately 2×10^9 cells/ml, the medium was further supplemented by addition of 0.5 liter of 8 mM Na_2SO_4, 0.94 M NH_4Cl, 16 mM $MgCl_2$, 36 μM $FeSO_4$. Cells were also grown on a "rich medium" which was this minimal medium supplemented with 1 g/liter of NaCl, 1 g/liter of Bacto-tryptone (Difco), and 0.5 g/liter of Bacto yeast extract (Difco). Strain MM1097 was grown and the λ*uncE114* phage was induced as described (11).

Cloning and Sequencing of uncE114 Gene—The 1.5-kilobase *Eco*RI/*Bam*HI fragment containing the *uncE* genes was cloned from λ*uncE114* into plasmid pBR322 essentially as described (11). This plasmid was digested with *Bam*HI and *Hpa*I, and the 435-base pair fragment containing the *uncE114* gene was cloned in opposite orientations in the multiple cloning site of M13mp18 and M13mp19 replicative form DNA for sequencing (17). The complete sequence of both strands of the cloned fragment was determined by the method of Sanger *et al.* (18), using single-stranded M13 phage DNA as template and a 15- or 17-base M13 primer (P-L Biochemicals).

Cell Fractionation and Membrane Preparations—The following buffers were used repeatedly in the procedures described below: TMDG, 50 mM Tris-HCl, pH 7.5, 5 mM $MgCl_2$, 1 mM dithiothreitol, 10% (v/v) glycerol; TEDG, 1 mM Tris-HCl, pH 8.0, 0.5 mM Na_2EDTA, 1 mM dithiothreitol, 10% (v/v) glycerol. The pH of both buffers was adjusted at room temperature. Cells were suspended in TMDG buffer containing 1 mM phenylmethylsulfonyl fluoride and 6 mM *p*-aminobenzamidine HCl and 0.1 mg/ml of DNase I and disrupted by passage through a French press at 18,000 p.s.i. at 4 °C. After centrifugation at 8,000 rpm (Sorvall SS34 rotor) at 4 °C for 15 min to remove large cell debris and unlysed cells, the low speed supernatant fraction was centrifuged at 186,000 × g_{max} at 4 °C for 75 min to collect the membrane fraction. The membrane fraction was resuspended in TMDG buffer containing 6 mM *p*-aminobenzamidine at 4 °C and centrifuged, and the washed membrane was stored in this buffer at −80 °C. In experiments where the distribution of ATPase activity between membrane and cytoplasmic fractions was determined, the low speed supernatant and high speed supernatant fractions were diluted with 0.2 volumes of 80% glycerol to stabilize the soluble ATPase and stored overnight at 0 °C prior to ATPase assay. Occasionally a portion of the membrane pellet sloughed off into the high speed supernatant fraction, and such supernatants were centrifuged a second time prior to assay. ATPase activity was assayed as described (19). Protein was determined by a modified Lowry assay containing SDS (19).

Membranes were stripped of F_1, and the crude F_1 preparations were concentrated and stabilized as described (20). F_1 binding to stripped membranes was determined essentially as described (15, 20). Stripped membrane (1 mg) and varying amounts of F_1 were incubated in 2 ml of TMDG buffer for 15 min at 30 °C and then overnight on ice. After centrifugation at 227,000 × g_{max} for 90 min, the tube and surface of the membrane pellet were rinsed with 2 ml of TMDG buffer and centrifuged again.[3] The rinsed pellet was resuspended in TMDG buffer and assayed for ATPase and protein.

ATP- and Respiration-dependent Quenching of Quinacrine Fluorescence—The method is essentially that described by Haddock and Downie (21), except that H_2O_2 and peroxidase were not included in the assay mixture. Varying amounts of membrane vesicles in 5–50 μl of TMDG buffer were added to 1.0 ml of 10 mM HEPES-NaOH, pH 7.5, 5 mM $MgCl_2$, 300 mM NaCl at room temperature. Assays were initiated with 5 μl of 0.15 mg/ml of quinacrine (~1.5 μM, final concentration) and the fluorescence was determined with an Aminco-Bowman fluorometer using 450 nm excitation and 510 nm emission. Further additions made to the cuvette were 10 μl of 0.1 M Na_2 ATP, 20 μl of 0.5 M Na_2 succinate, 5 μl of 10 mM or 10 μl of 0.1 M NADH, 10 μl of 2 mM DCCD in ethanol, 5 μl of 1 mM CCCP in ethanol.

Measuring Rates of H^+ Flux—The fluorometric procedure of Friedl *et al.* (22) was used with minor modifications. Stripped membrane vesicles (5 mg) were centrifuged and loaded with K^+ by resuspension in 5 ml of 250 mM K_2SO_4, 0.1 mM K_2EDTA, pH 8.0, at 40 °C. After 30 min, 0.5 ml of 0.1 M $MgSO_4$ was added and 10 min later 0.5 ml of 0.1 M MOPS-KOH, pH 6.8. The K^+-loaded vesicles were centrifuged (227,000 × g_{max}, 90 min at 4 °C) and the membrane pellet was stored on ice until immediately before assay. At this time the surface of the pellet was rinsed with 1 ml of 0.25 M Na_2SO_4, 5 mM $MgSO_4$, 25 mM MOPS-NaOH, pH 6.8, and resuspended in 0.25–0.5 ml of the same solution by homogenization.

The measuring device consisted of a mixing chamber connected by tubing to a peristaltic pump, a fluorimeter flow cell, and tubing returning to the mixing chamber. A solution of 3.2 ml of 25 mM MOPS-NaOH, pH 6.8, 0.25 M Na_2SO_4, 5 mM $MgSO_4$ was circulated through the system at 10.5 ml/min. ACMA (0.12 mg/ml in ethanol) was added to a final concentration of 0.8 μg/ml, followed by K^+-loaded membrane vesicles (0.4–3.6 mg). H^+ flux into the vesicles and the consequent quenching of ACMA fluorescence was initiated by addition of valinomycin (0.2 mg/ml in ethanol) to a final concentration of 1.25 μg/ml. Fluorescence was measured with a Gilson SpectroGlo fluorometer using a 330–400 nm excitation and 460–600 nm emission filter. ACMA was synthesized by and was a generous gift of Dr. C. P. Lee (Wayne State University).

Other Methods—Rates of succinate and NADH oxidation were measured with a Clark oxygen electrode utilizing a Yellow Springs meter and mixing chamber.

RESULTS

Genetics of uncE114 Indicate Single Mutation—Mutant strains carying the *uncE114* allele showed no growth on succinate minimal medium and gave a growth yield on 0.05% glucose that was 52% of the isogenic *unc*$^+$ control strain. Normal sized revertant colonies were formed on succinate minimal plates at a frequency of 3×10^{-6}, which is consistent with a single mutation. The *uncE114* mutation was originally assigned to the *E* complementation group based upon the positive complementation observed with λ*unc* transducing phage of the *B*, *F*, *A*, and *D* groups and lack of complementation by a λ*uncE* phage (11). A λ*uncE114* transducing phage was constructed and tested for complementation with recipi-

[3] For convenience the reconstituted membrane pellets were not resuspended by homogenization at this point since <5% of the ATPase in the reconstituted wild-type pellet was removed by this more extensive washing procedure.

ents of each of the eight defined complementation groups, inlcuding *H*, *G*, and *C*. A $B^+E^-F^+H^+A^+G^+D^+C^+$ pattern was indicated by the spot complementation test on succinate plates. The conclusion that the *E* gene was the sole defective gene was verified by a second type of complementation analysis. Strain MM994 (*uncE114*, *recA*) was complemented by plasmid pCP35 (*uncE*$^+$), which carries a fragment of the wild-type *unc* operon containing only the *uncE* gene (15). The pCP35/MM994 transformant produced colonies of normal size on succinate. This transformant gave a growth yield on 0.05% glucose that was 95% of that given by the isogenic *unc*$^+$ strain (MM833) and identical to the growth yield observed with the pCP35/MM833 (*unc*$^+$) transformant. These results indicate that the growth phenotype of *uncE114* can be ascribed solely to a mutation in the *uncE* gene.

Substitution Caused by the uncE114 Mutation—The genetic results reviewed above predict that the *uncE* gene should be altered by mutation. A 435-base pair fragment of DNA containing the *uncE* gene was cloned from λ*uncE114* and the DNA sequence was determined by the chain termination method of Sanger (18). A single substitution was detected, a C → G transversion at nucleotide 124 of the sense strand of the gene. The sequence of the remainder of the gene was identical to wild type. The mutation changes the codon from CAA to GAA and should cause a Glu for Gln substitution at residue 42 in the ω protein.

Distribution of ATPase Activity in uncE114 Mutant—Membranes prepared from strain MM973 (*uncE114*) retained near normal amounts of ATPase activity, based upon comparison of the specific activity of the membrane fraction to that from wild-type cells that were fractionated in parallel (Table I). The ratio of membrane-specific activities (MM973/MM180) ranged from 0.62–1.00 in three experiments. The mutant strain differed dramatically from wild type in that large amounts of ATPase were found in the cytoplasmic fraction, *i.e.* >5 times that normally observed. The high level of cytoplasmic ATPase in strain MM973 accounts for the near doubling of total activity observed in the cell-free lysate (low speed supernatant fraction). The difference in distribution of ATPase activity could suggest that the binding of F_1 to F_o is altered in the mutant.

Binding of F_1 to uncE114 Membrane—The bindings of wild-type F_1 to stripped membranes of strain MM973 (*uncE114*) and strain MM180 (*unc*$^+$) were compared. In two experiments with different membrane preparations, one of which is shown (Fig. 1), the concentration dependence of F_1 binding to the two types of stripped membrane was experimentally indistinguishable. However, as discussed below, the F_1 bound to MM973 membranes does under some conditions disassociate more readily than from wild-type membranes.

TABLE I

Amount and distribution of ATPase activity altered in uncE114 mutant

Strain	ATPase activity[a]		
	Low speed supernatant	High speed supernatant[b]	Washed membrane[b]
	$\mu mol \times min^{-1} \times mg^{-1}$		
MM180 (*unc*$^+$)	0.36 ± 0.02	0.12 ± 0.04 (20 ± 10%)	0.65 ± 0.10 (67 ± 10%)
MM973 (*uncE114*)	0.63 ± 0.14	0.65 ± 0.08 (65 ± 05%)	0.55 ± 0.17 (29 ± 12%)

[a] Average ± S.D. of three membrane preparations. The average recovery of protein in each fraction did not vary between wild-type to mutant strains.

[b] In parenthesis, average recovery of ATPase in this fraction relative to low speed supernatant (±S.D.).

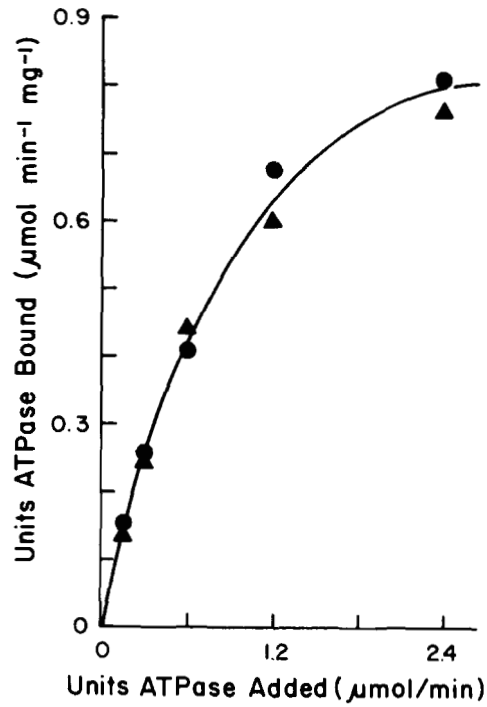

FIG. 1. **Binding of F_1 to stripped membranes of strains MM180 (*unc*$^+$) and MM973 (*uncE114*).** Variable amounts of F_1 were incubated with 1 mg of stripped membrane in 2 ml of TMDG buffer. The reconstituted membranes were collected by centrifugation, rinsed, and assayed for ATPase activity and protein content. ●, MM180 stripped membrane; ▲, MM973 stripped membrane.

H^+ Translocation by uncE114 F_o—Stripping of F_1 from wild-type membranes dramatically reduces respiration driven quenching of quinacrine fluorescence, the decrease usually being attributed to collapse of the ΔpH due to H^+ leakage through F_o. Stripped membranes from strain MM973 (*uncE114*) gave small quenching responses that were on the average somewhat greater than wild-type stripped membranes, the difference being reconsidered below. This property, which was also reported by Nieuwenhuis *et al.* (10), suggests most simply that the mutant stripped membranes are H^+-permeable due to exposure of the F_o H^+ channel. However, as discussed below, the extent of quenching varies with the relative rate of electron transport and cannot be related quantitatively to H^+ permeability.

In order to compare rates of F_o-mediated H^+ translocation by a means independent of electron transport, we have measured H^+ influx into vesicles in response to a K^+-diffusion potential using ACMA as a probe of ΔpH (Fig. 2). Both the initial rate and extent of quenching, after addition of valinomycin to establish the electrical potential, were nearly identical in *uncE114* and in *unc*$^+$ membrane vesicles over a range of membrane vesicle concentration (Figs. 2 and 3). The initial rate of fluorescence quenching was reduced 4–5-fold by DCCD, which indicated that H^+ flux was mediated primarily by F_o. The results of this more direct assay clearly indicate that the H^+-translocation function of the *uncE114* F_o was not significantly disrupted by the mutation.

Coupling of F_1 to F_o Disrupted in uncE114 Mutant—Several types of observations superficially suggest that the F_1 binds to the *uncE114* F_o in a manner where H^+ translocation is uncoupled from ATP hydrolysis. First, ATP hydrolysis by MM973 (*uncE114*) membrane vesicles did not generate a transmembrane pH gradient, as judged by the quenching of quinacrine fluorescence (not shown). Second, the ATPase

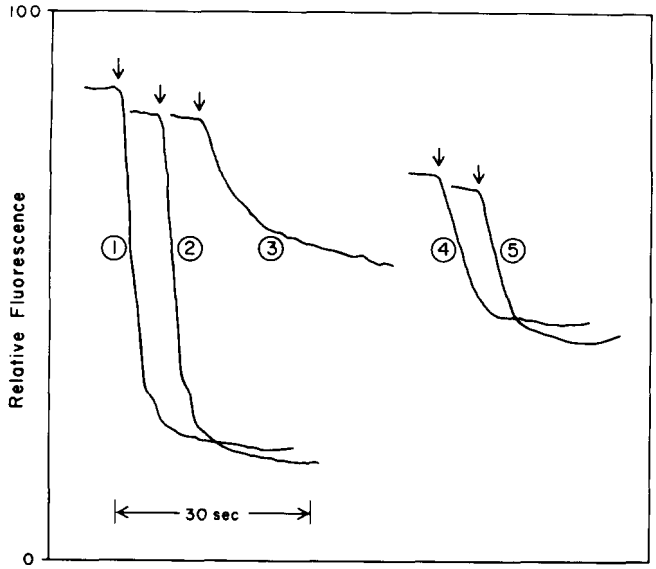

FIG. 2. **Valinomycin stimulated quenching of ACMA fluorescence comparing K^+-loaded stripped membrane vesicles from strains MM180 (*unc*$^+$) and MM973 (*uncE114*).** *Trace 1*, MM180 (3.6 mg); *trace 2*, MM973 (3.6 mg); *trace 3*, DCCD-treated MM180 (3.6 mg); *trace 4*, MM180 (0.4 mg); and *trace 5*, MM973 (0.4 mg). *Arrows* indicate point of mixing with valinomycin.

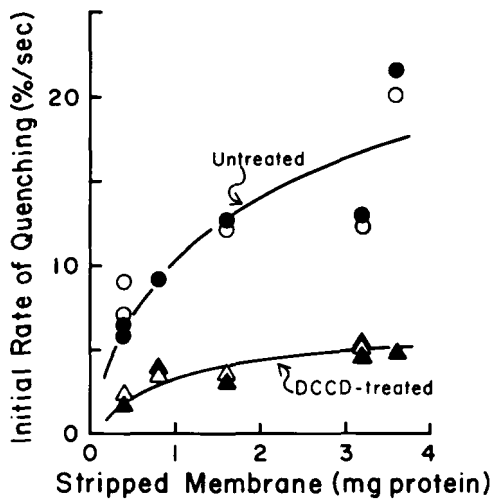

FIG. 3. **MM180 (*unc*$^+$) and MM973 (*uncE114*) stripped membrane vesicles show similar initial rates of valinomycin-stimulated ACMA quenching over a range of membrane protein concentration.** The initial rate of quenching of ACMA fluorescence is expressed as the rate of decrease in fluorescence/s normalized to the initial fluorescence prior to addition of valinomycin. ● and ▲, MM180; ○ and △, MM973. *Circles*, untreated stripped membranes; *triangles*, DCCD-treated stripped membranes. The results from two experiments using different membrane preparations are combined in the plot.

bound to the MM973 membrane was not inhibited by DCCD (not shown), even though DCCD reacts with F_o to block H^+ translocation (see above.).

The uncoupling of F_1 from F_o is also suggested by the high apparent H^+ permeability of MM973 membranes, which as shown above retain near normal levels of F_1-ATPase. The increased H^+ permeability, relative to wild type, is indicated by the small degree of succinate-driven quenching of quinacrine and the enhanced quenching observed after DCCD treatment (Fig. 4). However, this large difference between wild-type and mutant membranes in degree of quenching was

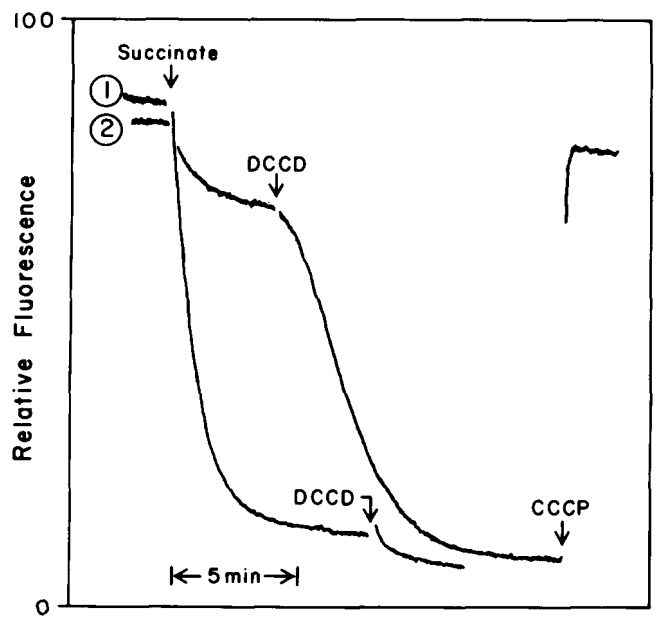

FIG. 4. **Succinate-dependent quenching of quinacrine fluorescence by MM180 (*unc*$^+$) and MM973 (*uncE114*) membrane vesicles.** *Trace 1*, MM180 whole membrane; *trace 2*, MM973 whole membrane. Both membrane preparations had a specific ATPase activity of 0.62 μmol × min^{-1} × mg^{-1}. Succinate, DCCD, and CCCP were added at the times indicated to concentrations of 10 mM, 20 μM, and 5 μM, respectively.

TABLE II

Comparison of respiration driven quenching of quinacrine fluorescence with whole membranes of wild-type and mutant

Substrate	Membrane preparation[a]	Quenching of fluorescence[b]			
		MM180 (*unc*$^+$)		MM973 (*uncE114*)	
		−DCCD	+DCCD	−DCCD	+DCCD
		%		%	
Succinate (10 mM)	M1 (500)	84	92	17	91
	M2 (220)	84	85	17	81
	(550)	76	82	49	85
	R1 (550)	32	34	7	61
NADH (50 μM)	M2 (220)	69		37	83
	(550)	67	70	36	
	R1 (550)	55	71	16	84
NADH (1 mM)	M1 (500)	82	83	79	85
	M2 (220)	84		67	90
	(550)	84	83	76	
	R1 (550)	78	84	53	88

[a] M1 and M2 denote two preparations of membranes prepared from cells grown on minimal medium. R1 denotes membrane preparation from cells grown on "rich" medium. The μg of membrane protein used per 1-ml assay is indicated in parenthesis.

[b] Maximal quenching expressed as per cent of initial fluorescence.

only observed under certain circumstances. The extent of the differences varied somewhat between membrane preparations, and was very dependent upon the amount of membrane used in the assay (Table II). The magnitude of the succinate-driven response for both wild-type and mutant membranes was greatly reduced when cells were grown on a rich medium (minimal medium supplemented with tryptone, yeast extract, and NaCl). When NADH was substituted for succinate as the substrate for electron transport at the concentration typically used in this assay (1 mM), the extent of quenching with MM973 (*uncE114*) membranes from cells grown on minimal medium differed only slightly from that of MM180 (*unc*$^+$) membranes (Table II). A larger difference in NADH-depend-

TABLE III

Comparison of rates of succinate oxidation by MM973 (uncE114) and MM180 (unc⁺) membranes

Membrane type	Rate of succinate oxidation	
	Minimal medium[a]	Rich medium[b]
	ng atom O × min⁻¹ × mg⁻¹	
MM180, whole	21.5–30.8	7.8
MM180, stripped	46.1–52.9	11.4
MM973, whole	26.0–39.1	12.2
MM973, stripped	50.4–66.5	19.0

[a] Range of two preparations.
[b] Single preparation.

ent quenching was observed when cells were grown on rich medium, or when a lower concentration of NADH (50 μM) was used in the assay (Table II). Finally, in many cases MM973 stripped membranes gave a larger quenching response than the nonstripped native membrane when equal amounts of protein were used in the assay (experiments not shown), which without further information suggested that the stripped membrane was less permeable to H^+ than the membrane with bound F_1.

At least part of the variability and unexpected differences in the magnitude of quenching may be attributable to differences in rate of succinate or NADH oxidation between membrane preparations. If the rate of electron transport coupled proton pumping is slowed, then the magnitude of the ΔpH generated (extent of quenching) should also be lessened.[4] On further examination, the rate of succinate oxidation was shown to differ significantly between wild-type and MM973 membranes, and was profoundly decreased by growth of cells on rich medium (Table III). The rate of succinate oxidation by stripped membranes averaged twice that of native membranes when normalized to mg of protein (Table III). Similar differences were seen in the relative rates of NADH oxidation.[5]

The above results suggested that it may be more appropriate to normalize degrees of quenching to rates of oxidation rather than mass of membrane protein. The extent of succinate-driven quenching did increase with the amount of membrane used in the assay for all membrane types with the significant exception of wild-type stripped membranes where a uniformly low quenching response was seen over the entire range (Fig. 5*A*). When the quenching response was normalized to oxidation rate (Fig. 5*B*), the MM973 whole membrane displayed greater quenching than the MM973 stripped membrane over the entire range. This suggested that the MM973 whole membrane was more H^+-permeable than wild type, but that the H^+ permeability was further increased by removal of F_1. Although the MM973 stripped membrane did show a low quenching response, it was marginally higher than wild-type stripped membrane over the entire range studied. The normalization of quenching to oxidation rate seems to be justified based upon the near coincidence of curves for the various types of DCCD-treated membranes (Fig. 5*C*). When the degree of quenching was normalized to the maximal quenching response, *i.e.* that given by DCCD-treated membrane, the relative response of each membrane type was nearly independent of membrane concentration over the range studied (Fig. 5*D*). In conclusion, this more detailed examination of the quenching response suggested an order of H^+ permeabilities with MM180 stripped membranes ≥ MM973 stripped membrane > MM973 whole membrane > MM180 whole membrane.

Explanation for H^+ Leakiness of uncE114 Membrane—The amount of ATPase activity released from MM973 membranes by EDTA stripping was comparable to that released from wild-type membranes. However, the crude F_1 preparation from the mutant was not as effective as wild-type F_1 in restoring function to wild-type stripped membranes, approximately 8 times more MM973 ATPase (units) being required to restore equivalent degrees of ATP-driven or succinate-driven quenching of quinacrine. This observation indicated that the F_1 bound to native MM973 membranes might be modified (*e.g.* by proteolysis), and this modification potentially could account for the H^+ leakiness of the MM973 membrane described above. This possibility was addressed in the following reconstitution experiment. Stripped membranes from MM973 (or the MM180 *unc*⁺ control) were incubated with an excess of wild-type F_1 (10 units/mg), and the reconstituted membranes were separated by centrifugation. Binding of F_1 resulted in a 5-fold enhancement in succinate-dependent quenching with the *unc*⁺ membrane but had no effect on the quenching response of the *uncE114* membrane (Fig. 6). The F_1 bound on the hybrid *uncE114* membrane was not modified since, on subsequent release from the membrane, it was bound again by stripped *unc*⁺ membranes and restored normal degrees of ATP- and NADH-driven quenching.[6] In summary, the F_1 found on native MM973 membranes is modified, either *in vivo* or as membranes are isolated from the cell, but this modification does not cause the uncoupling of F_1 from F_o and resultant increase in H^+ permeability.

The possibility that the high H^+ permeability of MM973 membranes was caused by disassociation of F_1 from F_o was examined. Kanner *et al.* (12) had reported that F_1 disassociated more readily from mutant K_{11} (*uncE114*) membranes than from wild-type membranes during washing with 50 mM Tris, pH 7.8, 10 mM $MgSO_4$ buffer. We also observed that the ATPase on MM973 native membranes was more readily solubilized during washing with TMDG buffer (15% *versus* 3% for wild-type membranes), although the extent of the loss was considerably less than that implied for the K_{11} mutant (12). The critical question was whether significant amounts of F_1 disassociated under the conditions used for assay of quenching of quinacrine fluorescence. When native MM973 or MM180 membranes were diluted into assay buffer (at 0.5 and 1.0 mg/ml of protein for both membranes), 25–26% of the MM973 ATPase activity was solubilized *versus* 4–5% for wild-type. Part of this difference can be attributed to the aforementioned modification of F_1. When similar measurements were done with MM973 or MM180 stripped membranes that had been

[4] Under the assay conditions used, the membrane potential is eliminated by the high chloride concentration. The net rate of H^+ translocation (dH^+/dt) should be related to the rate of pumping by the electron transport system, $(dO/dt)(H^+/O)$, and the leak rate governed by the effective H^+ conductance of the membrane (C_H) and magnitude of ΔpH, $dH^+/dt = (dO/dt)(H^+/O) - C_H\Delta pH$ (see Ref. 24). Under steady state conditions $dH^+/dt = 0$ and $\Delta pH = (dO)/dt)(H^+/O)/C_{H^+}$. The extent of quenching (Q) is presumed to be related to ΔpH (25), probably by a relationship where $Q/(1 - Q)$ is a function of ΔpH, and it should therefore vary with the rate of electron transport.

[5] The rate of NADH oxidation by MM973 membranes was 150–200% that of MM180 membranes. Stripped membranes gave specific rates of NADH oxidation 130–170% that of whole membranes.

[6] Wild-type stripped membranes were titrated with either fresh wild-type F_1 or wild-type F_1 that had been bound to and removed from MM973 stripped membranes. The titration curves were similar over a range of F_1 concentration that restored the quenching response by 20–90%. When normalized to ATPase activity, the F_1 that had been bound to MM973 stripped membranes was on average 94 and 103% as effective as the original F_1 in restoring the ATP-driven and NADH-driven quenching response, respectively.

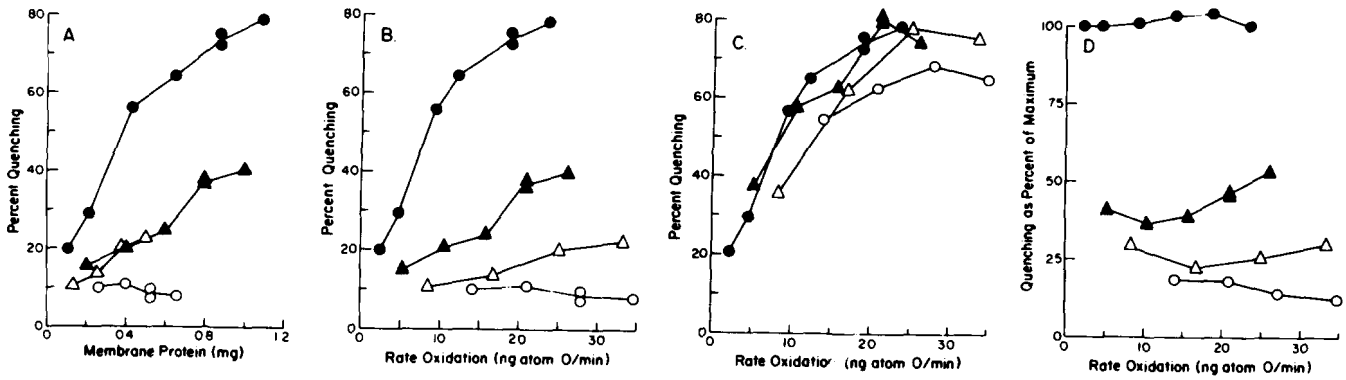

FIG. 5. **Dependence of succinate-driven quenching response on amount of membrane protein and rate of succinate oxidation.** *Symbols*: *circles*, MM180 whole (●) and stripped (○) membranes; *triangles*, MM973 whole (▲) and stripped (△) membrane. *A*, per cent quenching of quinacrine fluorescence *versus* amount of protein in assay. *B*, quenching response of untreated membranes related to rate of succinate oxidation. *C*, quenching response of DCCD-treated membranes related to rate of succinate oxidation. *D*, quenching response of untreated membranes normalized to the maximum quenching response seen with DCCD-treated membranes *versus* rate of succinate oxidation.

reconstituted with saturating levels of wild-type F_1, 15% of the ATPase activity was solubilized from the MM973 reconstituted membrane *versus* 6% for wild-type when these reconstituted membranes were diluted at 0.35 mg/ml into assay buffer. The question then addressed was whether disassociation of 15% of the F_1-ATPase from the membrane could account for the relatively high H^+ permeability exhibited by the reconstituted MM973 membrane (Fig. 6). Wild-type stripped membranes were reconstituted with varying amounts of F_1, and the amount of F_1 bound and per cent restoration of the quenching response were determined (Fig. 7). The relative quenching response was compared to that observed with MM973 stripped membranes and MM973 stripped membranes reconstituted with saturating levels of wild-type F_1 (Fig. 7). Binding of maximal amounts of F_1 to the MM973 stripped membranes membrane increased NADH-driven quenching somewhat (from 22 to 34% of that observed after DCCD treatment), but the extent of the quenching response remained much lower than that of wild-type membranes binding 66–74% of the saturating level of ATPase. Clearly, the observed disassociation of approximately 15% of the ATPase from the MM973 reconstituted membrane under conditions of assay cannot by itself account for the low quenching response.

DISCUSSION

We have concluded the following regarding the properties conferred by the *uncE114* mutation. 1) The H^+-translocating function of F_o is not disrupted. 2) F_1 binds to the *uncE114* F_o with near normal affinity, but the functional coupling between F_1 and F_o is disrupted. 3) The F_1 bound to isolated *uncE114* membranes is modified with partial loss of function, either *in vivo* or during preparation of membranes. 4) The modification of F_1 is not the cause of uncoupling between F_o and F_1. 5) All properties are due to the substitution of Glu for Gln at residue 42 of the ω subunit. The evidence bearing on these conclusions, both from the experiments presented here and earlier reports (10, 12), is evaluated below.

H^+ Translocation by Mutant F_o—The *uncE114* F_o clearly promotes H^+ translocation as was first indicated by the ACMA quenching experiments of Nieuwenhuis *et al.* (10). In the experiments reported here, stripped membranes from MM973 gave a low respiration-driven quenching response, but it was consistently somewhat larger than that given by wild-type stripped membranes. Part of the difference can be attributed to differences in rates of oxidation (Fig. 5). We presently have no means of quantitatively relating differences in extent of quenching to H^+ conductance. When rates of H^+-translocation were measured by a more direct means, *i.e.* H^+ flux in response to a K^+-diffusion potential, no significant difference between wild-type and mutant membranes was observed. This method of comparing H^+ conductance is certainly more quantitative and probably accurate to ±20%. The method may not be sensitive enough to pick up the small differences indicated by the respiration-driven quenching response. Conceivably, detection of the difference may be assay-dependent and not related to sensitivity. In the case of K^+-diffusion potential driven H^+ translocation, protons move from the F_1 binding side of the membrane to the interior of the inverted membrane vesicle. In the respiration-driven quenching studies, proton flux through F_o would be from the inside of the inverted vesicle to the outside to dissipate the ΔpH. Conceivably, the activity of the mutant F_o could be more influenced by the direction of $\Delta\psi$ or of H^+ flux.

Binding of F_1 to F_o—Kanner *et al.* (12) concluded that binding of F_1-ATPase to the membrane was altered in the K_{11} (*uncE114*) membrane, based upon the loss of ATPase from the membrane during washing with 50 mM Tris, pH 7.8, 10 mM $MgSO_4$ buffer. Using our routine cell fractionation procedure, we obtained washed mutant (MM973) membranes with ATPase activity nearly equivalent to wild-type. When these membranes were washed again with TMDG buffer, 15% of the ATPase activity was lost in the supernatant fraction *versus* 3% for wild-type membranes, which is considerably less than was implied to be lost from the K_{11} mutant (12). However, as discussed below, much of this difference appears to be due to a modified form of F_1 on the isolated MM973 membrane. In direct binding experiments, wild-type F_1 was shown to bind to MM973 stripped membranes with saturation kinetics that were identical to those of the wild-type control (Fig. 1). Clearly, the magnitude of difference in binding affinity must be relatively small.

F_1 from MM973 Membrane Modified—The F_1 removed from MM973 membranes was less efficient in restoring ATP-driven or succinate-driven quenching of quinacrine fluorescence to wild-type stripped membranes than the comparable F_1 preparation from wild-type membranes. Both preparations demonstrated equivalent ATPase activity. We interpret these results as indicating that the F_1 on MM973 membrane is

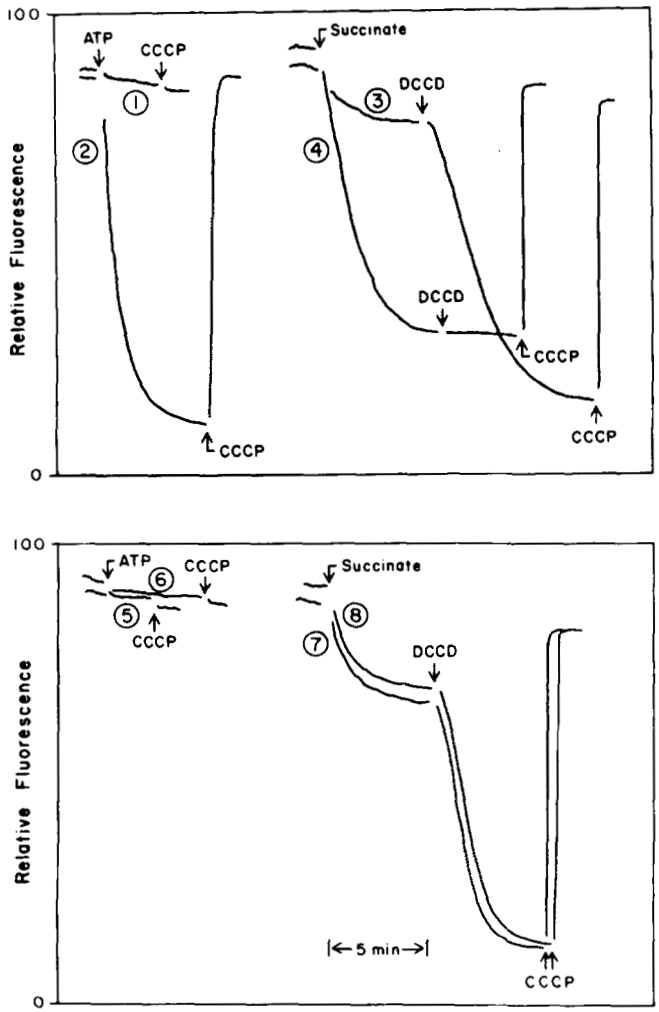

FIG. 6. **Comparative effects of binding wild-type F_1 to *unc*$^+$ or *uncE114* stripped membranes on ATP- and succinate-dependent quenching of quinacrine.** *Top panel*, MM180 (*unc*$^+$) stripped membranes before (*traces 1* and *3*) and after binding of F_1 (*traces 2* and *4*). *Lower panel*, MM973 (*uncE114*) stripped membranes before (*traces 5* and *7*) and after binding of F_1 (*traces 6* and *8*). The reconstituted membranes had specific ATPase activities of 1.08 μmol × min^{-1} × mg^{-1} (MM180) and 0.86 μmol × min^{-1} × mg^{-1} (MM973). Quinacrine was added to a suspension of 100 μg of stripped or reconstituted membrane in each experiment, and ATP, succinate, CCCP, or DCCD were added at the indicated *arrows*.

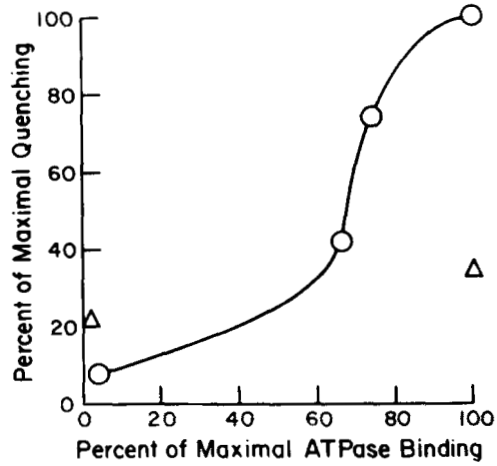

FIG. 7. **Relationship between extent of F_1 binding to stripped membranes and restoration of NADH-dependent quenching of quinacrine fluorescence.** MM180 or MM973 stripped membranes were incubated with varying amounts of wild-type F_1, centrifuged, washed, and assayed for ATPase activity and the quenching of quinacrine fluorescence elicited by 50 μM NADH. Since the NADH oxidase activity of the MM973 membranes was on the average 130% greater than MM180 membranes, and the amount of membrane protein used in the quenching assay varied somewhat (260–300 μg), the values have been normalized to the maximal quenching response observed after DCCD treatment (44% average for MM180 *versus* 55% average for MM973). The amount of ATPase bound has been normalized to the maximal level of binding (0.80 and 0.85 μmol min^{-1} mg^{-1} for MM180 and MM973, respectively). ○, MM180; △, MM973.

E. coli	Glu	Gly	Ala	Ala	Arg	Gln	Pro	Asp	Leu
PS-3	Glu	Gly	Ile	Ala	Arg	Gln	Pro	Glu	Leu
S. faecalis	Glu	Gly	Met	Ala	Arg	Gln	Pro	Glu	Met
M. lam.	Glu	Gly	Ile	Ala	Arg	Gln	Pro	Glu	Ala
Spinach	Glu	Gly	Ile	Ala	Arg	Gln	Pro	Glu	Ala
A. cald.	Glu	Gly	Val	Ala	Arg	Gln	Pro	Glu	Ala
N. crassa	Asn	Gly	Val	Ala	Arg	Asn	Pro	Ala	Leu
Bovine	Ile	Gly	Tyr	Ala	Arg	Asn	Pro	Ser	Leu
S. cerev.	Asn	Gly	Val	Ser	Arg	Asn	Pro	Ser	Ile
R. rubrum	Ser	Thr	Val	Gly	Arg	Asn	Pro	Ala	Ala

FIG. 8. **Amino acid sequence of residues Glu^{37} to Leu^{45} of *E. coli* ω subunit, and homologous sequences from DCCD binding protein of nine other species (see Ref. 8).** *Arrow* marks position of Glu for Gln^{42} substitution in *uncE114* mutant. Other species shown *top* to *bottom* are: thermophilic bacterium PS-3, *Streptococcus faecalis*, *Mastigocladus laminosus*, spinach chloroplast, *Acido caldarius*, *Neurospora crassa* mitochondria, bovine mitochondria, *Saccharomyces cerevisiae* mitochondria, and *Rhodospirillum rubrum*.

modified. On the other hand, Kanner *et al.* (12) were able to reconstitute ATP-driven quenching of ACMA fluorescence with the ATPase fractionating in the cytosolic fraction of disrupted K_{11} (*uncE114*) cells. In our hands, the supernatant fraction from MM973 cells showed no activity in this reconstitution test although it was enriched for ATPase activity (Table I). The reason for these differences in efficiency of reconstitution with different F_1 fractions remains unclear, except for the possibility that it may be strain-dependent.

The total ATPase activity (membrane plus cytosol) in disrupted cells of strain MM973 was on the average twice that observed for the isogenic wild-type strain. Kanner *et al.* (12) noted a similar increase in total ATPase activity (1.5–5-fold that of wild-type). Based upon the observation that both the membrane and cytosolic fractions show low to nil activity in reconstitutions with wild-type stripped membranes, we think it reasonable to suggest the following possible scenario. F_1 binds to the *uncE114* membrane with normal affinity, but abnormally in the sense that coupling between F_1 and F_o is lost. The abnormally bound F_1 is more susceptible to modification (most likely proteolysis), and modification reduces binding affinity and loss of F_1 to the cytoplasm. By unknown cellular controls the F_1 lost from the membrane is replaced by *de novo* synthesis, perhaps as a logical consequence of the dissimilar subunit stoichiometry in the final complex for subunits synthesized from a single operon (*e.g.* 3 α/10 ω) (26).

Bound F_1 Uncoupled from F_o—Unstripped membranes from *uncE114* mutants (K_{11} or MM973) are H^+ leaky as indicated by the extent of respiration-driven quenching of quinacrine or ACMA fluorescence. Based on the work reported by Nieuwenhuis *et al.* (10), the diminished succinate-dependent quenching response might have been ascribed to the loss of

F_1 from the membrane during preparation. In the studies reported here, mutant membranes retained up to normal amounts of ATPase but were still H^+ leaky. Further, the H^+ permeability of mutant stripped membranes was only slightly diminished by the binding of saturating amounts of wild-type F_1. Although more F_1 was lost from mutant *versus* wild-type membrane in the quinacrine quenching assay buffer, this difference in loss was not sufficient to account for the magnitude of the H^+ leak. The wild-type F_1 bound to mutant F_o in reconstitution experiments was not modified, and hence an altered form of F_1, as found on native *uncE114* membranes, cannot account for the H^+ leak. In conclusion, the H^+-leaky property of *uncE114* membranes is due primarily to an uncoupling between bound F_1 and F_o, and only in a minor way to an "open" F_o lacking F_1. The H^+ leak is one indication of the uncoupling, the others being DCCD-insensitive ATPase activity and the lack of ATP-driven quenching of ACMA (quinacrine) fluorescence (10), both properties being confirmed here.

Altered ω Subunit in uncE114 Mutant—The genetic complementation studies indicate that a single mutation accounts for the phenotype of the *uncE114* mutant, and a single substitution in the *uncE* gene was observed. The substitution results in a replacement of Glu for Gln at position 42 of the ω subunit. As shown in Fig. 8, the substitution occurs in the very conserved "polar loop" region of the protein. The striking conservation of sequence here led Sebald and Hoppe (7) to speculate that this region may be important to the functional interaction between F_1 and F_o. The consequence of the *uncE114* mutation, *i.e.* uncoupling without significant disruption of binding, support this idea and would most simply be interpreted as indicating that this region of ω lies on the F_1 binding side of the membrane. To date, this is the only evidence supporting this topological arrangement.

In summary, the phenotypic changes caused by the *uncE114* mutation differ strikingly from other F_o mutants that have been described. The F_o complex appears to be assembled normally, is functional in H^+ translocation, and binds F_1 with normal affinity. However, the functional coupling of F_1 to F_o is disrupted due to a single amino acid replacement in the conserved polar loop region of ω. It will be of interest to know whether other substitutions in this region have similar effects and whether the region plays a critical role in coupling H^+ translocation through F_o with the conformational changes in F_1 that are required for ATP synthesis (27).

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