A Mutation in the *Escherichia coli* F₀F₁-ATP Synthase Rotor, γE208K, Perturbs Conformational Coupling between Transport and Catalysis

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Cross-linking studies on the *Escherichia coli* F₀F₁-ATP synthase indicated a site of interaction involving γ and ε subunits in F₁ and subunit c in F₀ (Watts, S. D., Tang, C., and Capaldi, R. A. (1996) *J. Biol. Chem. 271*, 28341–28347). To assess the function of these interactions, we introduced random mutations in this region of the γ subunit (γ194–213). One mutation, γGlu-208 to Lys (γE208K), caused a temperature-sensitive defect in oxidative phosphorylation-dependent growth. ATP hydrolytic rates of the γE208K F₀F₁ enzyme became increasingly uncoupled from H⁺ pumping above 28 °C. In contrast, Arrhenius plot of steady-state ATP hydrolysis of the mutant enzyme was linear from 20 to 50 °C. Analysis of this plot revealed a significant increase in the activation energy of the catalytic transition state to a value very similar to soluble, ε subunit-inhibited F₁ and suggested that the mutation blocked normal release of ε inhibition of ATP hydrolytic activity upon binding of F₁ to F₀. The difference in temperature dependence suggested that the γE208K mutation perturbed release of inhibition via a different mechanism than it did energy coupling. Suppressor mutations in the polar loop of subunit c restored ATP-dependent H⁺ pumping and transition state thermodynamic parameters close to wild-type values indicating that interactions between γ and c subunits mediate release of ε inhibition and communication of coupling information.

F₀F₁-ATP synthases are found embedded in the inner membrane of mitochondria, the thylakoid membrane of chloroplasts, and the plasma membrane of eubacteria, and they harness the flow of protons down an electrochemical gradient to drive oxidative phosphorylation and photophosphorylation (for reviews, see Refs. 1–5). In *Escherichia coli*, the membrane-bound F₀ moiety contains three subunits, α, β, and ε, in a molar ratio of 1:2:9–12, and the soluble F₁ moiety contains five subunits, α, β, γ, δ, and ε, in a molar ratio of 3:3:1:1:1. Current models predict that H⁺ translocation in F₁ and catalysis in F₀ are linked via a rotational mechanism (6–9). In this model, the subunits are organized into “rotor” and “stator” elements. The rotor contains at least the γ-ε dimer from F₁ and possibly the subunit c oligomer from F₀ (6), and the stator contains the αβδ hexamer and δ from F₁ and subunits α and β from F₀. In the presence of an electrochemical gradient of protons, protons are carried through F₀ in a manner that generates a torque on the rotor (9). In turn, the torque drives cooperative ATP synthesis with all three catalytic sites participating (reviewed in Refs. 7 and 8).

There is little question that a rotary mechanism operates in the catalytic sector. The high resolution structure of the bovine mitochondrial F₁ (10) confirmed that alternating α and β subunits are arranged in a hexamic ring and revealed that the amino and carboxyl termini of the γ subunit form an α-helical coiled-coil that projects into the αβδ hexamer. A unique face of the γ subunit imposes an asymmetry on the β subunits and the catalytic sites. This asymmetry is necessary for cooperative catalytic turnover as the αβδ hexamer is symmetric (11) and has no site-site cooperativity (12) in the absence of the γ subunit. The direct observation of ATP hydrolysis-dependent rotation of the γ subunit relative to the αβδ hexamer demonstrated that rotation is a mechanistic feature of cooperative catalysis (13). A model of the reaction pathway (14) suggests that the three different conformations of each β subunit observed in the crystal structure represent the major ground states that each site passes through during a catalytic cycle. As the γ subunit rotates, it drives each of the three sites through its cycle offset from the others by 120°, and specific conformational information is transmitted between the γ and β subunits which is critical for energy coupling (15, 16).

Although the x-ray crystallographic structure provided a great deal of information about the catalytic sector of the enzyme, it did not contain the part of the rotor that connects catalysis to transport. Electron density was missing for the middle two-thirds of the γ subunit and the entire ε subunit (in *E. coli* nomenclature; this subunit is called δ in the mitochondrial complex). Nevertheless, solution structures of isolated *E. coli* ε and c subunits have been obtained by NMR (17–20). The c subunits contain two transmembrane α-helices bridged by a polar loop (17–19). Situated in the middle of the carboxy-terminal helix within the membrane bilayer lies cAsp-61, a conserved carboxylic acid believed to be directly involved in proton transport. Dicyclohexylcarbodiimide modification of cAsp-61 blocks both H⁺ transport and ATP hydrolysis (21) and induces conformational changes in the polar loop region (17). The loop region is exposed to the cytoplasm and has been implicated in F₁ binding in a manner that is important for energy coupling (22–26). Mosher et al. (22) first found a functional interaction between F₀ and F₁ by isolating a mutation in the polar loop of subunit c, cQ42E, that uncoupled the enzyme. This notion was confirmed when second-site mutations were identified in the ε subunit (εE31G, V, and K) which suppressed the deleterious effects of cQ42E (27). They later found that εE31C can be induced to form disulfides with Cys replacements in subunit c at positions 40, 42, and 43 (28). Interestingly, Cys
replacements of almost the same residues, 42, 43, and 44, also formed disulfides with γY205C (29). Watta et al. (29) also showed that γY205C efficiently forms disulfides bonds with eH38C or eT43C. These cross-links had little effect on ATP hydrolysis, reinforcing the notion that e subunit is a part of the rotor. As one might expect, eGlu-31, eHis-38, and eThr-43 are on the same side of a 10-stranded β-barrel created by the amino-terminal half of the subunit (20). The β-barrel also contains a region that has been suggested to impose partial inhibition of ATPase activity in the soluble F1 complex (ε59–70 as suggested by Xiong and Vik (30) or ε80–93 as suggested by Kuki et al. (31)). This inhibition is released upon binding of F1 to F0 (15, 32).

The results described above suggest a critical site of interaction between γ, ε, and c subunits. To assess the functional role of the interactions at the γ-ε-c interface, we screened random mutations in the region of the γ subunit near γY205. We describe here the effects of a particularly interesting mutation, the replacement of γGlu-208 with Lys. Thermodynamically, the mutant F1,F1 enzyme acted like F1 inhibited by ε subunit but also had a temperature-sensitive defect in oxidative phosphorylation and energy coupling. The effects of the γE208K1 mutation were suppressed by second-site mutations near the polar loop of subunit c but not by mutations in the ε subunit. These findings establish a functional relationship between the γ and c subunits and suggest that the interaction between these subunits mediates the release of ε inhibition and plays a role in the transmission of coupling information between catalysis and H+ transport.

**EXPERIMENTAL PROCEDURES**

**Strains and Plasmids—**The expression plasmid, pACWU1.2 (33), carries the eight structural genes for the F0,F1 complex and complements the unc operon-deleted E. coli strain, DK8 (h2R, thi-1, rel-1, HfrPO1, ΔuncB-uncC) ilc::Tn10; Ref. 34). To assist mutagenesis and cloning, several subclones were used. pBluescript II KS+ (Stratagene) and contains the entire uncG (γ subunit gene) between KpnI and SpeI. pE208K1 was derived from pUC18 and contains a portion of uncD (β subunit gene) and the entire uncC (ε subunit gene) between EcoR1 and XhoI. pBH-RI and pBH-RII was derived from pBlueScript II KS+ (Stratagene) and contains most of uncE and all of uncF, E, and H (a, c, b, and δ subunit genes, respectively) between BamHI and EcoRI.

**Molecular Biology Procedures—**Molecular biology procedures were performed according to manufacturers’ instructions or as detailed by Sambrook et al. (35). Restriction and DNA-modifying enzymes were obtained from Amersham Pharmacia Biotech, Boehringer Mannheim, Life Technologies, Inc., New England Biolabs, and Promega. A Stratagene Chameleon Kit was used for oligonucleotide-directed mutagenesis (36). “Dirty test tube” oligonucleotides were synthesized by Oligos Etc. (Wilsonville, OR) using 97% fidelity. In other words, they were obtained from Amersham Pharmacia Biotech, Boehringer Mannheim, Life Technologies, Inc., New England Biolabs, and Promega. A Stratagene Chameleon Kit was used for oligonucleotide-directed mutagenesis (36). “Dirty test tube” oligonucleotides were synthesized by Oligos Etc. (Wilsonville, OR) using 97% fidelity. In other words, they were obtained from Amersham Pharmacia Biotech, Boehringer Mannheim, Life Technologies, Inc., New England Biolabs, and Promega. A Stratagene Chameleon Kit was used for oligonucleotide-directed mutagenesis (36). “Dirty test tube” oligonucleotides were synthesized by Oligos Etc. (Wilsonville, OR) using 97% fidelity. In other words, they were obtained from Amersham Pharmacia Biotech, Boehringer Mannheim, Life Technologies, Inc., New England Biolabs, and Promega. A Stratagene Chameleon Kit was used for oligonucleotide-directed mutagenesis (36). “Dirty test tube” oligonucleotides were synthesized by Oligos Etc. (Wilsonville, OR) using 97% fidelity.

**Growth of Strains and Membrane Preparations—**Strain DK8 harboring derivatives of pACWU1.2 was grown on supplemented minimal medium as described previously (38, 39). For determination of oxidative phosphorylation-dependent growth, 0.4% sodium succinate was used as a sole carbon source (38). To prepare F1,F1-containing membrane vesicles, strains were grown until mid-log in minimal medium containing 1.1% glucose at 37 °C, and membranes were isolated as described previously (40). Protein concentrations were determined by the method of Lowry et al. (41).

**Purification of F1—**Purification of soluble F1 was carried out using an epitope tag. The sequence encoding the Flag epitope (DYKDDDDK) was introduced at the 5′ end of uncD (δ subunit gene) in both pACWU1.2

<table>
<thead>
<tr>
<th>Preparation</th>
<th>Range of F1 content (% F1)</th>
<th>Growth on succinate (mg of F1)</th>
</tr>
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<tbody>
<tr>
<td></td>
<td>25 °C</td>
<td>37 °C</td>
</tr>
<tr>
<td>DK8/pACWU1.2</td>
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<td></td>
</tr>
<tr>
<td>Wild-type membranes</td>
<td>9–12</td>
<td>100</td>
</tr>
<tr>
<td>γE208K membranes</td>
<td>9–10</td>
<td>60</td>
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</tbody>
</table>

*Range of F1 associated with membrane preparations expressed as percent of total membrane protein. Determination of F1 content in membrane preparations was performed by a quantitative immunoblot analysis described under “Experimental Procedures.”

Growth yield in 0.4% liquid sodium succinate medium.

and pACWU1.2/γE208K as described previously (33). F1 was dissociated from 20 mg of membranes and precipitated with polyethylene glycol 8000 (42, 43). The crude soluble F1 containing the Flag epitope was applied to a 1-ml anti-Flag M2 affinity column (Kodak), pre-washed, and packed as suggested by the manufacturer. The column was washed with 10 volumes of a buffer containing 50 mM Tris, 10% v/v glycerol, 1 mM MgCl2, 1 mM ATP, 40 mM 6-aminohexanoic acid, and 150 mM NaCl, at pH 7.4. Purified F1 complex was eluted with four sequential washes of 0.25 mg/ml Flag peptide (DYKDDDDK) in wash buffer containing 40 mM Na2SO4 instead of 150 mM NaCl. The yield was 0.5–1 mg of F1 and purity was judged to be >95% based on Coomassie staining of a SDS-polyacrylamide gel.

**Determinations of F1 Content—**Determination of F1 content in membrane preparations was performed by a quantitative immunoblot assay described previously (15). Purified F1 was used as a reference standard.

**F1 Depletion and Reconstitution—**In order to strip away the F1 moiety, 100 μg of membrane protein was added to 1 ml of a buffer containing 1 mM Tris, 10% v/v glycerol, and 0.5 mM EDTA and stirred at room temperature for 15 min. The F1-depleted membranes were pelleted by centrifugation at 54,000 × g at 4 °C for 30 min, and the procedure was repeated. Reconstitution was carried out by adding a 2-fold excess of purified F1 to stripped membranes and incubating with stirring at 37 °C for 30 min.

**Enzymatic Assays—**ATP hydrolysis rates and formation of an electrochemical gradient of protons were determined as described previously (15, 44). For Arrhenius analysis, temperature dependence of ATPase activity was measured in buffers containing 50 mM HEPES-KOH, 10 mM ATP, and 5 mM MgSO4; the pH was adjusted to 7.5 at the appropriate temperature (15). The concentration of free Mg2+ and Mg-ATP was calculated using the algorithm of Fabiato and Fabiato (45).

**RESULTS**

The γE208K Mutation Causes a Temperature-sensitive Defect in Oxidative Phosphorylation-dependent Growth—To assess the role of the interactions between subunits at the F0,F1 interface, random mutations were introduced into a region of the E. coli γ subunit (residues 194–213) that was demonstrated by cross-linking of cysteine replacements to interact with the ε subunit in F1 and the c subunits in F0 (28, 29, 46). Dirty test tube synthesis was employed to randomly introduce approximately one base change per oligonucleotide (see “Experimental Procedures”). These oligonucleotides were used as primers in a standard site-directed mutagenesis protocol, and the mutagenized DNA fragment was shotgun cloned into the unc operon carried on plasmid pACWU1.2 (33). The mixture of plasmids was used to transform strain DK8, and the transformants were screened for oxidative phosphorylation-dependent growth with sodium succinate as a sole carbon source. Seven mutant strains were initially selected based on poor growth at 37 °C. One mutation in particular, γE208K, proved interesting because it caused a temperature-sensitive growth defect. The γE208K mutant grew to 60% of wild-type at 25 °C but only 3% at 37 °C (Table I). The γE208K mutation did not appear to disturb complex assembly because the content of F1 associated with the isolated membrane vesicles was not different from wild-type membranes (Table I). This observation argued that the mutation affected growth by perturbing the functionality of...
the complex and reduced the rate of coupled ATP synthesis.

The γE208K Mutant Enzyme Is Uncoupled above 28 °C—To understand better the temperature-sensitivity of the γE208K mutant, we measured rates of ATP hydrolysis, ATP-driven H+ pumping, and NADH-driven H+ pumping over a range of temperatures from 7 to 40 °C. As seen in Fig. 1A, the rate of ATP hydrolysis and ATP-driven H+ pumping of the mutant enzyme increased relative to wild type up to about 28 °C, however, above that temperature ATP hydrolysis continued to increase while ATP-driven H+ pumping decreased. The decreased H+ pumping was not due to a passive leak of protons because the γE208K enzyme maintained the same level of NADH-driven H+ pumping as wild type. Clearly, catalysis became uncoupled from transport. The proportionate rates of ATP hydrolysis and ATP-driven H+ pumping of the γE208K mutant enzyme became apparent in a plot of the ratio of relative ATP hydrolysis and ATP-driven H+ pumping (Fig. 1B). At temperatures up to 28 °C, the given rate of ATP hydrolysis generated the expected degree of ATP-driven H+ pumping for a coupled enzyme. Above 28 °C, the ratio of ATP hydrolysis and ATP-driven H+ pumping increased dramatically indicating that catalysis and H+ transport became increasingly unlinked with rising temperature.

If the γE208K mutation perturbed the γ-ε subunit interface and did not permanently alter F0 function, then F0 from the γE208K mutant complex should reconstitute with wild-type F1 to form a complex with wild-type characteristics. We stripped the F1 moiety from both γE208K and wild-type membranes (in both cases <1% of the original ATPase activity remained after stripping) and reconstituted each with either purified γE208K or wild-type F1. Indeed, wild-type F1 reconstituted with stripped γE208K membranes had the same level of ATP-driven H+ pumping as the wild-type F1 reconstituted with stripped wild-type membranes (Table II). As expected, H+ pumping by the γE208K F1 reconstituted with either stripped γE208K or wild-type membranes was considerably lower than the wild-type control. These results indicated that the defect in coupling was due to the γE208K amino acid change and not due to an irreversible defect in the assembly of the F0 sector.

ε Subunit Inhibition of γE208K Mutant F1 Is Not Relieved

### Table II

<table>
<thead>
<tr>
<th>Membrane preparation</th>
<th>Source of purified F1</th>
<th>% WTε</th>
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</thead>
<tbody>
<tr>
<td>Stripped wild-type membranes a</td>
<td>γE208K F1</td>
<td>47</td>
</tr>
<tr>
<td>Stripped γE208K membranes b</td>
<td>Wild-type F1</td>
<td>100</td>
</tr>
<tr>
<td>Stripped γE208K membranes b</td>
<td>Wild-type F1</td>
<td>41</td>
</tr>
<tr>
<td>Stripped γE208K membranes b</td>
<td>Wild-type F1</td>
<td>101</td>
</tr>
</tbody>
</table>

a The 100% value was the extent of acridine orange quenching obtained from the stripped wild-type membrane reconstituted with wild-type F1.
b NADH-dependent H+ pumping at 37 °C for both wild-type and γE208K F1-depleted membranes was 7 and 9%, respectively, compared with unstripped wild-type membranes.

c When Bound to F0—Transition state thermodynamic parameters of the ATP hydrolytic reaction previously have been shown to be highly sensitive to alterations in protein conformation and subunit-subunit interactions (14, 15). Arrhenius analysis of steady-state ATP hydrolysis was performed in Vmax conditions with an ATP-regenerating system and a protonophore to prevent back inhibition from build up of ADP or ΔΔG°, respectively. From these data, it is apparent that the γE208K enzymes, whether as the membranous F0F1 or the soluble F1, have very similar temperature dependence to the wild-type F1 (Fig. 2).

From the Arrhenius plots, the transition state thermodynamics parameters were derived and are listed in Table III. First, we note that both the wild-type and γE208K F1 were purified by means of a Flag epitope tag added to the amino terminus of the β subunit (33). The Flag sequence did not alter the transition state structure, as the thermodynamic parameters of the tagged F1 were similar to those previously reported for “normal” F1 (15). Second and most significantly, both enthalpic and entropic transition state thermodynamic parameters for the γE208K F0F1 and F1 were similar to wild-type F1 but had much more positive values compared with wild-type F0F1 (Table III).

A well known feature of the soluble F1 enzyme is the inhibition of cooperative ATP hydrolytic activity by the ε subunit (47). As in the case of wild-type F1, dilution of γE208K F1 to 0.1 nm, well below the Kd for binding of ε subunit to F1 (15, 32), resulted in activation of the ATPase activity (Fig. 3) and indicates that ε subunit interacts normally with the γE208K F1. We have previously confirmed (15) that the inhibition is normally released upon binding of F0 to F1 (32). Here we observe that there is little difference between γE208K F0F1 and F1 transition state thermodynamic parameters and turnover rates and that these parameters are very close to the wild-type F1. These results strongly suggest that the effect of the γE208K mutation is to block release of the ε inhibition upon F0 binding to F1. These data also suggest that the normal difference between F1 and F0F1 transition state thermodynamic parameters (Table III) can be attributed to ε subunit inhibition.

At this point, we note that the failure to release the ε subunit inhibition in the γE208K F0F1 appeared to be a different effect from the temperature-dependent uncoupling shown in Fig. 1. The clearly linear Arrhenius plot between 20 and 50 °C for the mutant represented the transition state structure of the ε-inhibited enzyme, whereas there was no obvious effect over the temperature range at which the enzyme became uncoupled.

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**Fig. 1. Temperature dependence of γE208K mutation. A. ATP hydrolysis (■), ATP-driven H+ pumping (○), and NADH-driven H+ pumping (●) of γE208K membrane vesicles expressed as a percent of wild-type over a range of temperatures from 7 to 40 °C. All three assays were performed in a buffer containing 10 mM HEPES-KOH, 300 mM KCl, 5 mM MgCl2, 1 μM valinomycin, and 1 μM acridine orange at pH 7.5. B. data from A plotted as the ratio of ATP hydrolysis and ATP-driven H+ pumping (both relative to wild type) as a function of temperature.**
Energy Coupling at the F₀F₁ Interface

Fig. 1. Arrhenius analysis of wild-type and ϵ208K-purified F₁ and membranous F₀F₁ enzymes. Log of the turnover rates (s⁻¹) is plotted against the reciprocal of absolute temperature as detailed under “Experimental Procedures.” The lines plotted were generated by linear least squares regression of the data. ATPase activities for purified wild-type F₁ (○) and ϵ208K F₁ (●) were assayed from 5 to 45 °C, and ATPase activities for membranous wild-type F₀F₁ (▲) and ϵ208K F₀F₁ (●) were assayed from 20 to 50 °C. The arrow indicates 28 °C above which the ϵ208K F₀F₁ became uncoupled. As previously reported, the Arrhenius plot for membranous F₀F₁ has a transition around 19 °C but is linear above this temperature (15). The transition is due to an effect of F₀ on F₁ and is likely a manifestation of the influence of the lipid phase on the function of the F₀ which is communicated to the catalytic mechanism through coupling.

Table III
Transition state thermodynamic parameters at 37 °C comparing wild-type and ϵ208K membranous F₀F₁ and purified F₁

<table>
<thead>
<tr>
<th>Preparation</th>
<th>Turnover ratea</th>
<th>ΔH′</th>
<th>ΔS′</th>
<th>ΔG′</th>
<th>ΔG′F₁</th>
<th>ΔG′DD</th>
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<tbody>
<tr>
<td>DK5/pACWU1.2</td>
<td>s⁻¹</td>
<td></td>
<td></td>
<td></td>
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<td>Wild-type membranes</td>
<td>471</td>
<td>34.6</td>
<td>-25.6</td>
<td>60.2</td>
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<tr>
<td>ϵ208K membranes</td>
<td>216</td>
<td>55.5</td>
<td>20.9</td>
<td>66.6</td>
<td>19.0</td>
<td>62.1</td>
</tr>
<tr>
<td>Wild-type F₁</td>
<td>208</td>
<td>56.6</td>
<td>-5.7</td>
<td>62.3</td>
<td></td>
<td></td>
</tr>
<tr>
<td>ϵ208K F₁</td>
<td>175</td>
<td>56.4</td>
<td>-0.2</td>
<td>63.0</td>
<td>-0.6</td>
<td>62.7</td>
</tr>
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</table>

aATPase assays were performed in a buffer containing 50 mm HEPES-KOH, 10 mm ATP, 5 mm MgSO₄, pH 7.5, with 5 mm phosphoenolpyruvate and 32 μg/ml pyruvate kinase as an ATP-regenerating system. 5 μM carbonyl cyanide m-chlorophenylhydrazone was included with the membrane assays to ensure no back inhibition from ΔμFₒ.

bTurnover numbers were calculated using a molecular mass of 3.8 × 10⁶ daltons for the F₁ complex.

cΔΔ values are differences between mutant enzyme and the corresponding wild-type enzyme preparations (membranes compared to membranes, and F₁ compared to F₁).

dValues for ΔG′DD were derived utilizing turnover rate determinations at 37 °C.

Fig. 2. Activation of the ATP hydrolytic activity upon dilution and c subunit release. Purified ϵ208K F₁ (open bars) or wild-type F₁ (solid bars) were diluted to the specified concentrations, incubated at 37 °C for 30 min, and then assayed for ATPase activity in the same buffer as in Table III. The error bars indicate one standard deviation (n = 3).

Second-site Mutations within the c Subunits Restore Oxidative Phosphorylation-dependent Growth—The proximity of the γ, ε, and c subunits near γGlu-208 indicated by cross-linking experiments (28, 29, 46) led us to hypothesize that the ϵ208K mutation caused altered interactions between these subunits. To test this hypothesis and for functional interactions between subunits, we searched for second-site mutations that suppressed the deleterious effects of ϵ208K. Again, the dirty test tube synthesis of mutagenic oligonucleotides was used to introduce random mutations in two regions as follows: 1) the β-barrel domain of the c subunit between amino acids 28 and 45, and 2) the middle section of subunit c between amino acids 34 and 51. After mutagenesis was carried out as described above, we screened for return-of-function mutants by selecting for oxidative phosphorylation-dependent growth at 37 °C.

Out of approximately 10⁴ clones derived from the random mutagenesis of the c subunit, no strains were found capable of forming a colony on a succinate minimal medium plate. In contrast, several second-site suppressor mutations were identified in subunit c. DNA sequencing of the mutagenized region revealed that five of the strains had single amino acid changes, and two had two changes (Table IV). In each case, a DNA fragment containing the mutation was ligated into the original pACWU1.2, wild type, or with ϵ208K and tested again for growth. Table IV lists the growth yields in 0.4% liquid succinate medium at 37 °C for each of the second-site suppressor mutations expressed either alone or in conjunction with ϵ208K. Each of the subunit c mutations caused increased growth yield of the ϵ208K mutant strain, and interestingly, none of the suppressor mutations alone had an appreciable effect (growth yields listed in parentheses). The effect of cA39G and cL45V on the oxidative phosphorylation-dependent growth of the ϵ208K mutant appeared to be additive in that the growth of the double mutant, cA39G/L45V + ϵ208K, was roughly the sum of the two single mutants. The mutations of the other double mutant, cL36V/cD44G, were not tested separately.

The suppressor mutations caused varying degrees of increased turnover rates of ATP hydrolysis compared with the ϵ208K enzyme. The F₁F₁ steady-state ATP hydrolytic activity of each of the suppressor mutations in combination with ϵ208K were subjected to Arrhenius analysis, and the transition state thermodynamic parameters were derived (Table V). In every case, the transition state enthalpic and entropic parameters were much closer to the wild-type enzyme. The reduced differences in the transition state parameters correlated with the increased turnover rates of ATP hydrolysis, consistent with partial or complete relief of c subunit inhibition.

Interestingly, the suppressor mutations fell into two distinct...
groups in their capacity to restore ATP-driven H⁺ pumping and to maintain NADH-driven H⁺ pumping as follows: the first group, cK34N, cK34R, and cL46S, did not restore in vitro H⁺ pumping to the γE208K enzyme and caused the most notable passive leaks of protons as assessed by NADH-driven H⁺ pumping, whereas the second group, cL36M/D44G, cA39G, and cL45V, restored ATP-driven H⁺ pumping to near wild-type levels and caused little or no passive leaks. The first group of mutants may have suppressed the deleterious effects of γE208K. The gray circles indicate γ, ε, and c subunit residues that form inter-subunit disulfide bonds when changed to cysteines (see Introduction and “Discussion”).

**TABLE IV**

<table>
<thead>
<tr>
<th>Strain</th>
<th>Range of F₁ content</th>
<th>Growth on succinate</th>
<th>Turnover rates of ATP hydrolysis</th>
<th>ATP-driven H⁺ pumping</th>
<th>NADH-driven H⁺ pumping</th>
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<tbody>
<tr>
<td>DK8/pACWU1.2 membranes</td>
<td>% F₁</td>
<td>% WT</td>
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<tr>
<td>γE208K</td>
<td>9–10</td>
<td>3</td>
<td>46</td>
<td>19</td>
<td>100</td>
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<tr>
<td>cK34N + γE208K</td>
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<td>20 (99)</td>
<td>60</td>
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<td>cK34R + γE208K</td>
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<td>27 (91)</td>
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<td>cL36M/D44G + γE208K</td>
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<td>27 (91)</td>
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<td>cA39G/L45V + γE208K</td>
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<td>81 (95)</td>
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<td>97</td>
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<td>cL45V + γE208K</td>
<td>8–11</td>
<td>52 (99)</td>
<td>92</td>
<td>96</td>
<td>99</td>
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</table>

* Growth yield in 0.4% liquid sodium succinate minimal medium for each second-site suppressor mutation expressed alone is given in parentheses.

**TABLE V**

Differences in transition state thermodynamic parameters at 37 °C between wild-type and γE208K membranes plus second-site suppressor mutations

Wild-type and γE208K transition state thermodynamic parameters at 37 °C are as listed in Table II.

<table>
<thead>
<tr>
<th>Strain</th>
<th>ΔΔH²⁰</th>
<th>ΔΔTS²⁰</th>
<th>ΔΔG²⁰</th>
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<tbody>
<tr>
<td>DK8/pACWU1.2 membranes</td>
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<td></td>
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</tr>
<tr>
<td>γE208K</td>
<td>20.9</td>
<td>19.0</td>
<td>1.9</td>
</tr>
<tr>
<td>cK34N + γE208K</td>
<td>3.8</td>
<td>2.7</td>
<td>1.1</td>
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<td>cK34R + γE208K</td>
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<td>−1.3</td>
<td>0.5</td>
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<td>cL36M/D44G + γE208K</td>
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<td>2.3</td>
<td>−1.2</td>
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<td>−7.4</td>
<td>−0.1</td>
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<tr>
<td>cA39G/L45V + γE208K</td>
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<td>1.1</td>
<td>1.0</td>
</tr>
<tr>
<td>cL45V + γE208K</td>
<td>0.8</td>
<td>0.6</td>
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<tr>
<td>cL46S + γE208K</td>
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<td>0.4</td>
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* ΔΔ values are differences between parameters for mutant enzyme and wild type.
different mechanisms, the same primary mutation, γE208K, disrupted both coupling and regulation of ε inhibition. Furthermore, the same second-site mutations suppressed both defects. It is likely that γE208K uncoupled the enzyme at non-permissive temperatures by disrupting a specific interaction between γ and ε subunit or by altering the conformation of the γ subunit at the F₀-F₁ interface. These results clearly demonstrate the communication of conformational information between γ and ε subunits.

Fillingame and co-workers (22–26) have established by analysis of numerous mutations that important conformational information is transmitted through the polar loop of subunit c to F₁, including specific interactions between ε and c subunits (27). Here, we demonstrate that there are also important functional interactions between γ and c subunit. The second-site mutations that suppress γE208K map to two discrete regions of subunit c. Based on the NMR structure of the c subunit in chloroform:methanol:water² (17), the first region was on the amino-terminal side of the polar loop between amino acids 34 and 39 (εK34N, εK34R, and εA39G, see Fig. 4). The second region was toward the carboxyl-terminal side of the polar loop between amino acids 44 and 46 (εL45V and εI46S). The γE208K mutation was also suppressed by two double mutants, εL36⁷/D44G and εL45V/I46S, both of which contained a replacement on either side of the positions (40–44) where cysteine residues were found to cross-link to γT205C and εE31C (28, 29).

Several suppressor mutations found in discrete regions of the c subunits may suggest that the γ subunit, including γ-Glu-208, interacts with the c subunits next to the polar loop and slightly into the membrane bilayer (Fig. 4). There is also the possibility that the c subunit suppressor mutations act at a distance from γE208K through conformational effects; however, the cross-linking results suggest that these residues are in close proximity. In addition, based on results from Watts and Capaldi (49), the γ subunit may be interacting with multiple c subunits. Via conformational linkage, the γE208K mutation may affect packing of the subunit c oligomer, which could perturb transport function by affecting the environment of cAsp-61.² These possibilities are currently under investigation.

We also note the differences between γE208K and another γ subunit uncoupling mutation previously characterized, γM23K. Both mutations have similar effects on transition state thermodynamic parameters; however, the γM23K mutation affected γ-β interactions, whereas γE208K affected γ-ε interactions. The γM23K mutation creates an additional bond between γ and β subunits, which is revealed by the increased transition state thermodynamic parameters in both F₀F₁ and F₁. On the other hand, only γE208K F₀F₁ appears to have an altered transition state, an effect which we have assigned to the failure to release the bond causing ε inhibition. We previously showed that the γM23K mutations caused a Kₘₑₐₓ defect because it created a branched pathway in the catalytic cycle (14). Because γE208K appears to perturb the functional linkage to transport, it will likely be a Vₘₑₐₓ defect.

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² M. Girvin, personal communication.
A Mutation in the *Escherichia coli* F$_1$-ATP Synthase Rotor, γE208K, Perturbs Conformational Coupling between Transport and Catalysis

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