Energy Coupling, Turnover, and Stability of the F₀F₁ ATP Synthase Are Dependent on the Energy of Interaction between γ and β Subunits*

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Replacement of the F₀F₁ ATP synthase γ subunit Met-23 with Lys (γM23K) perturbs coupling efficiency between transport and catalysis (Shin, K., Nakamoto, R. K., Maeda, M., and Futai, M. (1992) J. Biol. Chem. 267, 20835–20839). We demonstrate here that the γM23K mutation causes altered interactions between subunits. Binding of δ or ε subunits stabilizes the αβγ complex, which becomes destabilized by the mutation. Significantly, the inhibition of F₁ ATP hydrolysis by the ε subunit is no longer relieved when the γM23K mutant F₁ is bound to F₀. Steady state Arrhenius analysis reveals that the γM23K enzyme has increased activation energies for the catalytic transition state. These results suggest that the mutation causes the formation of additional bonds within the enzyme that must be broken in order to achieve the transition state. Based on the x-ray crystallographic structure of Abrahams et al. (Abrahams, J. P., Leslie, A. G. W., Lutter, R., and Walker, J. E. (1994) Nature 370, 621–628), the additional bond is likely due to γM23K forming an ionized hydrogen bond with one of the βGlu-381 residues. Two second site mutations, γQ269R and γR242C, suppress the effects of γM23K and decrease activation energies for the γM23K enzyme. We conclude that γM23K is an added function mutation that increases the energy of interaction between γ and β subunits. The additional interaction perturbs transmission of conformational information such that ε inhibition of ATPase activity is not relieved and coupling efficiency is lowered.

The F₀F₁ ATP synthase links two disparate functions: transport of protons across a membrane and catalysis of ATP synthesis or hydrolysis (for reviews see Refs. 1–5). The fully cooperative mechanism of ATP hydrolysis requires a minimum of three different subunits in a complex containing αβγ1. The transport mechanism is most likely assembled from F₀ sector subunits. In the Escherichia coli complex, transport requires three different membrane-spanning subunits, αβγδ ε−10 (6). In addition, two more soluble subunits, δ and ε, are needed to reconstitute catalytic and transport sectors so that they are coupled to carry out ATP-driven proton pumping or ΔμH⁺-driven ATP synthesis (7–10).

Catalysis and transport mechanisms most likely communicate indirectly through a series of conformational and electrostatic interactions. Conformational changes relevant to the catalytic state of the enzyme or the presence of a ΔμH⁺ have been detected by several methods including altered cross-linking patterns, protease susceptibility, environmentally sensitive fluorescent probes, accessibility of epitopes, x-ray diffraction, cryoelectron microscopy, and spectroscopic analyses (reviewed in Refs. 11 and 12). High resolution structural information based on crystals of the bovine mitochondrial F₁ has also provided a great deal of information about possible subunit interactions that may be involved in linking transport and catalysis (13).

Mutagenic analysis has also yielded important information about the coupling mechanism (reviewed in Ref. 5). For example, mutations in the single hydrophilic loop of subunit c, an F₀ subunit that is involved in proton transport, disrupt coupling between transport and catalytic mechanisms (14–16). Furthermore, genetic and chemical cross-linking results strongly suggest that c subunit interacts with this portion of subunit c (17, 18).

Likewise, mutations near the catalytic sites have also been found to affect coupling. The most clear example of these mutations is replacement of γMet-23 with Arg or Lys (19). These mutations caused greatly reduced ATP-dependent proton pumping and ATP synthesis rates without strongly affecting catalytic or transport functions. The F₀ sector was unaffected as were interactions between F₀ and F₁. Restoration of efficient coupling in the γMet-23 → Lys (γM23K) mutant enzyme was conferred by several second site mutations near the carboxyl terminus of the γ subunit including the replacement of amino acid γArg-242 and seven different residues between γGln-269 and γAla-280 (20). Furthermore the temperature sensitivity caused by the γM23K mutation was suppressed by each of the second site mutations. In the simplest interpretation of these results, direct interactions within the γ subunit are perturbed by the γM23K mutation, and each of the suppressor mutations is close enough to the γM23K residue to directly counteract its influence on structure and function. However, many observations do not coincide with this interpretation (21). Analysis of the x-ray crystallographic structural map of the bovine F₁ (13) indicates that γMet-23 is a considerable distance from the γ269–280 region. Furthermore, the types of amino acid changes that suppress the effects of the γM23K mutant are not of any particular functional group, and in some cases multiple changes at a single site result in suppression (20, 21). It is difficult to imagine how multiple and diverse amino acid replacements would be able to compensate for a specific perturbation.

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Because the mechanism of coupling must involve interactions among subunits, we hypothesized that the γM23K mutation perturbed interactions between subunits. In this paper, we provide evidence that the γM23K mutation is an added functional mutation that increases the energy of interaction between γ and β subunits. The consequences of this increase are destabilization of the αβγ complex and inefficient coupling between transport and catalysis. In turn, the suppressor mutations described above counteract by decreasing the energy of interaction.

MATERIALS AND METHODS

Strains and Plasmids—F₁ complex and δ and ε subunits were isolated from strain DK8 harboring plasmid, pBWU13 (22) or pBMU13-γM23K (see below). Membranous F₁Fₒ was obtained from strain KF10A harboring derivatives of plasmid pBWG15 (23).

The γM23K mutation was introduced into the aceG gene on plasmid pBWU13 to give pBMU13-γ-M23K. Oligonucleotide-directed mutagenesis with the Stratagene (La Jolla, CA) Chameleon Kit (24) was used to introduce the γM23K mutation on plasmid pBWG11 (23) using the synthetic oligonucleotide, 5'-CACTAAGAGCAAGATGGTGGCCC-3' (lowercase letters denote the γM23K mutation). After sequence verification, a mutated fragment was isolated on the Ausil II to Rsr II restriction fragment and ligated into pBWU13. Introduction of the mutation into the expression plasmid and presence of the mutation after growth were verified by phenotype and DNA sequencing (25).

Molecular biological manipulations were performed as described (26) or according to the manufacturer’s instructions. Restriction enzyme and DNA modifying enzymes were obtained from Amersham Corp., Boehringer Mannheim, Life Technologies, Inc., New England Biolabs, (Beverly, MA), or Promega (Madison, WI).

Isolation of Membranes and Purification of F₁ and F₁ Subunits—Membranes from KF10A were prepared as described previously (8). F₁ complex was purified as detailed by Duncan and Senior (27) and Al-Shawi and Senior (37). Purified wild-type F₁ was used as a source for the expression plasmid and presence of the mutation after growth were verified by phenotype and DNA sequencing (25).

Enzymatic Assays—Protein concentrations were determined by the method of Lowry et al. (31). ATPase activities were measured in the buffers given below and in the figure legends by procedures described in Al-Shawi et al. (32). The experimental conditions detailed in the footnotes to Table II were chosen to optimize for coupling efficiency and enzyme stability. Free Mg²⁺ and Mg-ATP concentration were determined by the method of Fabiato and Fabiato (33). ATPase reactions were stopped by the addition of 5% sodium dodecyl sulfate or 10 mM ice-cold H₂SO₄. Liberated Pₐ was determined by the methods of Tauskky and Shorr (34) or Van Veldhoven and Maanaerts (35) depending on the sensitivity required. The Van Veldhoven and Maanaerts assay was slightly modified by stopping the final color development reaction with the addition of 0.5 M H₂SO₄ after 20 min of incubation at room temperature. ATP synthesis was measured as described previously (36). Pyruvate kinase was obtained from Boehringer Mannheim, and hexokinase was from Sigma.

Arrhenius Analysis and Derivation of Transition State Thermodynamic Parameters—Apparent enzyme activation energies of ATP hydrolysis were calculated from measurements of maximal rates of ATPase activities as a function of temperature. Activation energies and entropic and enthalpic components of the transition state for ATP hydrolysis were calculated from plots of log velocity at saturating ATP (in the presence of an ATP regenerating system) versus the reciprocal temperature as detailed in Al-Shawi and Senior (37).

RESULTS

The γM23K Mutation Does Not Alter ε or δ Subunit Interactions with F₁—We first assessed if the γM23K mutation altered interactions with the single copy F₁ subunits, δ and ε. In the wild-type E. coli complex, ε subunit binds tightly to the F₁ complex with a Kᵦ of approximately 10⁻⁶ M (38), and the association constant of isolated γ and ε subunits is similar to this value (39). These results suggested that ε interactions with the remainder of the F₁ complex are mostly through the γ subunit. In turn, the affinity for ε subunit to γ subunit can be assessed by the inhibitory properties of the ε subunit on ATPase activity. ATPase activity of the αβγε complex is inhibited approximately 90% by ε subunit; the Kᵦ is very close to the binding constant between isolated γ and ε subunits (39).

Sternweis and Smith (38) showed that dilution of the purified F₁ complex to a concentration below the Kᵦ for ε-F₁ results in activation of ATPase specific activity. Fig. 1 reproduces this result where the enzyme is activated as F₁ is diluted below 100 nM. The same activation is observed for wild-type and γM23K enzymes. Titration of the wild-type F₁ indicates a half-maximal activation at approximately 10 nM, which is the same value as previously reported (9, 38). δ subunit is generally believed to bind F₁ with lower affinity than ε subunit and is most likely dissociated at the concentration of 10 nM F₁ (40).

In a marked difference from wild type, further dilution of γM23K F₁, caused inactivation of activity with half-maximal inactivation occurring at 0.2 nM. The inactivation was not detected with the wild-type F₁, even at the lowest F₁ concentration measured, 0.01 nM. This indicates that the γM23K mutation causes destabilization of αβγε, the minimum complex capable of ATPase activity (32, 41).

Interestingly, the inactivation of the γM23K enzyme does not occur until ε and δ subunits have dissociated and suggests that binding of ε and δ subunits may help to stabilize the complex. To test this notion, superstoichiometric amounts of purified ε or δ subunits were added, while γM23K F₁ was diluted to various concentrations (Fig. 2). In the presence of 72 nM ε subunit alone, the ATPase specific activity of 13 nM γM23K F₁ (the concentration that gave maximal activation) was decreased as expected due to ε inhibition. At 1.3 nM γM23K F₁, the ATPase specific activity remained about the same, and at 0.13 nM γM23K F₁, the activity increased more than 2-fold compared with the absence of added ε subunit. This behavior reflects the balance between the dissociation/association of ε subunit and inactivation of αβγε enzyme complex.

Results with 72 nM δ subunit were even more dramatic because the activity was stabilized even at the most dilute F₁ concentrations. The activities at the two lower concentrations were maximal, indicating that the concentration of ε subunit was well below its Kᵦ. Clearly, δ subunit alone binds to the complex independent of the ε subunit and in a manner that stabilizes αβγε. With both δ and ε subunits added, activity was

1The abbreviation used is: CCCP, carbonylcyanide-m-chlorophenylhydrazone.
consistent with ε-inhibited levels.

The stabilization of activity by δ subunit alone provided a way to directly assess the $K_I$ for ε subunit. γM23K F₁ was diluted to 0.13 nM in the presence of 72 nM δ subunit to maintain the stabilized complex. Titration of ε subunit resulted in an apparent $K_I$ of 13 nM (data not shown), which is in good agreement with the dilution experiments in Fig. 1.

γM23K F₁ Does Not Release ε Inhibition upon Binding to F₀—In order to more fully analyze the kinetic and thermodynamic properties of the γM23K enzyme, we accurately determined the concentration of F₀,F₁ complex in the membrane of E. coli strain KP10rA. This was done by a quantitative immunoblot analysis described under “Materials and Methods.” In brief, the amount of immunostaining obtained for each of the membrane preparations was compared with the amount of staining of known amounts of purified F₁ loaded on the same gel (data not shown).

Knowing the amount of F₁ on the membranes, we were able to derive turnover numbers for native F₀,F₁ in membranes. Fig. 3 compares the turnover numbers for wild-type and γM23K enzymes as soluble F₁ or membranous F₀,F₁. The wild-type F₀,F₁ turnover was 425 s⁻¹ (at 30 °C) compared with 92 s⁻¹ for soluble F₁, which is about 85% replete with the inhibitory ε subunit (Figs. 1 and 2). This result confirms the early work of Sternweis and Smith (38) that ε inhibition is relieved upon binding to F₀. Interestingly, the turnover numbers of the γM23K F₁ and F₀,F₁ are quite similar (132 s⁻¹ and 92 s⁻¹, respectively).
Two important observations are to be made from these values. First, the γM23K mutation does not affect the catalytic mechanism because wild-type and the γM23K enzymes have relatively similar turnover numbers, and second, the ε inhibition of the γM23K enzyme is not relieved when bound to F₀. The latter results may indicate that the γM23K mutation perturbs the functional interaction of F₁ with F₀ as mediated by the ε subunit.

**Altered Transition State Thermodynamic Parameters in the γM23K Mutant Enzyme**—In order to understand the effects of the γM23K mutation, we investigated the results of the mutation on the catalytic mechanism under steady state conditions. We already saw that the turnover of the γM23K F₀F₁, was similar to that of wild type. Furthermore, both enzymes had a pH optimum of around 8.5–9.0 as well as the Kₘ values for Mg-ATP hydrolysis being similar (0.16 and 0.32 mM for wild-type and γM23K F₀F₁, respectively; data not shown). These results reinforce the conclusion that the general reaction schemes and cooperative mechanisms of the mutant enzyme were similar to those of the wild-type enzyme. Additional support for this conclusion can be seen later in the “isokinetic” plots (see Fig. 8) in that the mutant enzyme preparations were close to the regression lines.

However, we have previously demonstrated that the catalytic transition state of the F₀F₁ ATP synthase is very sensitive to changes in catalytic site conformation and the utilization of binding energy to drive catalysis (42). Thus, in order to probe the effects of the γM23K mutation on catalysis and coupling, we measured the thermodynamic parameters of the transition state of ATP hydrolysis by Arrhenius analysis of steady state turnover. In contrast to the results above, the activation energies for the catalytic transition state were strongly affected. Fig. 4 shows an Arrhenius plot of steady state ATPase activity (for clarity, log of the actual ATPase specific activities are plotted instead of turnover). Temperature dependence of maximal velocities were measured with 5 mM Mg-ATP. In the case of membrane-bound enzymes, the protonophore, carbonyl cyanide-m-chlorophenylhydrazone, was added to prevent back inhibition from the electrochemical gradient of protons. Purified F₁ from wild type and γM23K had linear plots from 5 to 45 °C, whereas the membrane-bound F₀F₁ had a break in the plot around 19 °C. The break in the Arrhenius plot is clearly due to an effect of F₀ on F₁. This effect 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. Significantly, the change in the slope for γM23K F₀F₁ is much less pronounced than that for the wild-type enzyme, suggesting that the influence of F₀ on catalysis is decreased in the mutant complex.

From these plots the enthalpic, entropic, and free energy terms can be calculated for the transition state of the reaction pathway. These values for the wild-type and γM23K F₁ at 30 °C are listed in Table I and plotted in Fig. 5A. The differences for each parameter between wild type and γM23K are plotted in Fig. 5B. Note that the membrane-bound enzyme had the same trends as soluble F₁, but the γM23K F₀F₁ had considerably larger differences in both enthalpic and entropic terms. For both soluble and membrane-bound complexes, the γM23K enzyme has a more positive ΔH° and a less negative ΔS° which together add up to a small difference in ΔG°. According to transition state theory, these results suggest that the mutation causes the formation of additional bonds between substrate and enzyme or, more likely in this case, bonds within the enzyme that must be broken in order to achieve the transition state.

**Suppressor Mutations of γM23K Reverse the Effects on Activation Energies**—If the changes in transition state thermodynamic parameters are due to the same perturbations that causes the uncoupling phenotype of the γM23K mutation, then second site suppressor mutations of γM23K (20) would be expected to reverse the altered thermodynamic parameters. This was the case with the suppressor mutation, γQ269R.

This mutation was most effective at restoring efficient coupling. The ratio of NADH-driven ATP synthesis versus ATP hydrolysis can be used as a parameter of coupling efficiency (21, 43). Table II shows that the synthesis:hydrolysis ratio of the γM23K mutant is restored to wild-type levels in the presence of γQ269R. As predicted, the Arrhenius analysis shows that the γQ269R mutation counteracted the effects of the

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**Table I**

| Preparation  | ΔH°  | ΔΔH° | TΔS° | ΔTΔS° | ΔG°  | ΔΔG° |
|--------------|------|------|------|-------|------|------|
| Wild-type F₁ | 51.0 | -11.8| 10.7 | 22.5  | 62.9 | -0.9 |
| γLys-23 F₁  | 72.7 | 21.6 |       |       |      |      |
| Wild-type membranes | 31.3 | -27.8| 59.0 |       |      |      |
| γLys-23 membranes | 76.8 | 45.5 | 14.0 | 41.7  | 62.9 | 3.8  |

ΔΔ values are differences between parameters for mutant enzyme and the corresponding wild-type preparation. See legend to Fig. 4 for assay conditions.
γM23K mutation on the thermodynamic parameters of the ATPase transition states (Fig. 6).

In contrast, another suppressor mutation, γR242C, did not increase the coupling efficiency even though it genetically suppressed the γM23K mutation (20) and resulted in increased ATP synthesis rates (Table II). Instead, the mutation suppressed the effects of the γM23K mutation by increasing the turnover rate of the enzyme by 1.44-fold. This result is reminiscent of our observations that overexpression of the γM23K F_{0}F_{1} resulted in increased ATP synthesis rates and increased yields on the nonfermentable carbon source, succinate (20). With more enzyme present, albeit an inefficient one, there was sufficient ATP synthesis to allow growth of the cell.

Consistent with the lack of recovery of efficient coupling, differences in the transition state thermodynamic parameters of the γM23K mutant were only slightly reduced by introduction of γR242C (Fig. 6). In this case, it appears that the reduced energy of interaction between γ and β subunits resulted in a faster turnover rate.

## DISCUSSION

We have sought to understand how the γM23K mutation perturbs linkage between transport and catalysis to provide information about the mechanism of coupling. We have seen that the mutation does not affect F_{1} interactions with ε and δ subunits (this report) nor the F_{0} sector (19); however, dissociation of δ and ε subunits leaves a destabilized α_{βγ} complex (Fig. 1). Based on chemical cross-linking experiments, ε subunit is believed to interact with γ, β, and α subunits (44–47) and apparently does so with a stabilizing effect on the α_{βγ} complex. An important property of the ε subunit is its inhibitory activity on the hydrolysis activity of F_{1}. Most significantly, in contrast to the situation in wild type, inhibition by ε subunit in the γM23K mutant is not relieved upon binding to F_{0} (Fig. 3), suggesting a perturbation in communication between transport and catalytic mechanisms. This notion was supported by the decreased change in slope of the Arrhenius plot for the membranous γM23K enzyme (Fig. 4). Both of these effects illustrate the impaired coupling between F_{1} and F_{0} functions in the mutant enzyme γM23K.

From the above data, it seems that the γM23K mutation affects the regions of interactions between γ and β subunits. Based on suppressor mutagenesis results, we earlier concluded that three highly conserved regions of the γ subunit, γ18–35, γ238–246, and γ269–280, functionally interact as a domain that mediates energy coupling (21). Upon close inspection of the x-ray crystallographic structural model of Abrahams et al. (13), we observed that each of these three regions is in contact with the surrounding β subunits and that at least two of the residues form a hydrogen bond with specific β subunit residues, namely γGln-269 with βThr-304 and γArg-242 and βGlu-381 (see below and Fig. 7A). It is apparent that effects of γ subunit mutations in these regions on catalysis are due to perturbation of the interactions with the catalytic β subunits. Thermodynamic analysis of transition state activation energies for ATP hydrolysis reactions strengthen this notion. Significantly, γM23K mutant enzyme had dramatically more positive ΔH and less negative ΔS parameters, suggesting that the amino acid replacement caused an extra bond to form that must be broken in order to achieve the catalytic transition state.

Fig. 7B shows details of the bovine F_{1} structural map. γM23 is a member of a conserved triad consisting of γR242 and one of the three βE381. The illustrated βE381 is in the β subunit conformer known as βD, which has ADP bound in the F_{1} crystal (13). We note that all three residues are in highly conserved regions of the γ and β subunits and are identical in all known γ subunit sequences; therefore, in the analysis of mutant E. coli complexes, using the positions of these residues as determined from crystals of the bovine enzyme is valid. In turn, we note that the results presented here are entirely consistent with the structural model of Abrahams et al. (13). We propose that when γM23 is changed to lysine, the ε-amino group forms an additional ionized hydrogen bond with βGlu-381 during a step of the catalytic cycle, hence the extra bond detected by Arrhenius analysis. Clearly, γM23K is an added function mutation. This conclusion is consistent with the similar uncoupling effect of replacing γMet-23 with arginine and the lack of effect when substituted by neutral or negatively charged amino acids (19).

This explanation is also consistent with the effects of two site mutations that suppress the effects of the γM23K mutation. The γQ269R suppressor mutation restored efficient coupling and negated the increase in transition state activation energy. As mentioned before, the amino acid replacement of γGln-269 affected the interactions between γ and β subunits, causing reduced coupling efficiency (Table II) and stability (20). We propose that the γQ269R mutation suppresses the effect of γM23K in part by reducing the energy of interaction between γ and β subunits. It is likely that similar effects were observed by Jeanfeu-D De Beukelaer et al. (48) with β subunit mutations that suppressed the effects of an altered γ subunit carboxyl terminus. Related to the effect on coupling is an effect on complex stability. Loss of δ and ε subunits leaves a destabilized

### Table II

| Strain | ATP synthesis* | ATP hydrolysis* | Coupling ratio |
|--------|----------------|----------------|---------------|
| Initial rate | % of Wild type | Rate | % of Wild type | Synthesis/hydrolysis | Ratio % of Wild type |
| KF10rA/pBMG15 | 0.024 | 0.029 | 0.06 | 0.32 | 31 |
| Wild type | 0.24 | 0.29 | 0.56 | 0.32 | 31 |
| γLys-23 | 0.052 | 0.29 | 0.62 | 0.10 | 31 |
| γCys-242 | 0.06 | 0.24 | 0.50 | 0.35 | 109 |
| γCys-242 + Lys-23 | 0.06 | 0.24 | 0.50 | 0.35 | 109 |
| γArg-269 | 0.047 | 0.24 | 0.50 | 0.35 | 109 |
| γArg-269 + Lys-23 | 0.047 | 0.24 | 0.50 | 0.35 | 109 |

* ATP synthesis rates were measured at 37°C with vigorous shaking in a buffer containing 25 mM HEPES-KOH, 200 mM KCl, 5 mM MgSO_{4}, 10 mM glucose, 1 mM ADP, 10 mM [γ^{32}P]Pi, 50 units/ml hexokinase, and 0.1–0.2 mg/ml E. coli membranes. The reactions were started with the addition of 2 mM NADH. Control blank rates were obtained under the same conditions in the presence of 5 mM free Mg^{2+}, 10 mM glucose, 1 mM ADP, 10 mM [γ^{32}P]Pi, 50 units/ml hexokinase, and the reactions were started with the addition of 0.08–0.2 mg/ml E. coli membranes. Time points were taken up to 20 min, and the samples were analyzed as described under “Materials and Methods.”

ATP hydrolysis rates were measured at 37°C in a buffer containing 25 mM HEPES-KOH, 200 mM KCl, 5 mM MgSO_{4}, 10 mM glucose, 1 mM ADP, 10 mM [γ^{32}P]Pi, 50 units/ml hexokinase, and the reactions were started with the addition of 2 mM NADH. Control blank rates were obtained under the same conditions in the presence of 5 mM free Mg^{2+}, 10 mM glucose, 1 mM ADP, 10 mM [γ^{32}P]Pi, 50 units/ml hexokinase, and the reactions were started with the addition of 0.08–0.2 mg/ml E. coli membranes. Time points were taken up to 6 min, and the samples were analyzed as described under “Materials and Methods.”
Fig. 6. Effect of suppressor mutations on transition state thermodynamic parameters of γM23K F_0F_1. Difference activation energy parameters for membranous F_0F_1 preparations (mutant enzyme minus wild-type enzyme values) were obtained at 30 °C by Arrhenius analysis as detailed in the legend to Fig. 4. Thermodynamic values were calculated as described under “Materials and Methods.” Thermodynamic values for F_0F_1 preparations containing individual γ subunit mutations were compared with those for F_0F_1 preparations containing double γ subunit mutations (the original γM23K mutation in conjunction with a “suppressor” mutation). Values for membranous F_0F_1 preparations containing the γM23K mutation are represented by the hatched bars.

α_3β_3γ with γM23K (Fig. 1), and all of the known suppressor mutations of γM23K confer temperature stability (20, 21). Clearly, protein-protein interactions between γ and β subunits are critical for both complex stability and coupling.

In contrast, changing γR242C resulted in overall higher proton pumping rates and ATP synthesis because the enzyme turned over faster and not because coupling efficiency was restored. Interestingly, the structure suggests that γR242C should reduce the energy of interaction between γ and β subunits because this amino acid change should remove the ionized hydrogen bond between γArg-242 and βDP Asp-381 (Fig. 7B). It is possible that the cysteine may reduce γ-β interactions even more if the environment of γR242C induces a lower pK and causes the residue to ionize at neutral pH. The thiolate ion, a strong nucleophile, would form an ion pair with γM23K and in addition create a repulsive pair with βGlu-381. We are analyzing the properties of complexes with additional mutations of both residues to clarify their roles. Not surprisingly, certain amino acid replacements of βGlu-381 have a similar uncoupling phenotype to the γM23K mutation.3 Without question, the γ-β interactions involving γR242 and βE381 play an important role in turnover and coupling. The β subunit residue is a part of the conserved sequence (E380DELSEED386 (E. coli numbering)) near the “hydrophobic sleeve” (13). The lower asterisk shows the contact region between the conserved residue γGln-269 and βThr-304 (E. coli numbering) near the “hydrophobic sleeve” (13). The upper asterisk shows the contact region between the conserved residue γArg-242 and βGlu-381 of the conserved β 380DELSEED386 sequence in βGlu. This region is shown in detail in B along with γMet-23 and Van der Waals' contacts.

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gested that the mitochondrial enzyme is a "better" catalyst for energy. In order to achieve a lower transition state, however, the enzyme binds the substrate in a manner that is closer to the true transition state of pentacoordinated $\gamma$- mutants. This is the primary effect of $\gamma$- M23K on coupling. Another interesting feature revealed by the isokinetic plots (Fig. 8) is that the $\gamma$M23K $F_1$ and $\gamma$M23K $F_0F_1$ points have very similar values, whereas the wild-type $F_1$ point has a more positive $\Delta H^\ddagger$ and $\Delta S^\ddagger$ than the wild-type $F_0F_1$ membrane preparations. The origin of this phenomenon was seen in Fig. 3 in that as the wild-type $F_1$ binds to $F_0$, the $\epsilon$ inhibition is relieved on binding $F_0$. As pointed out above, the effect of the $\gamma$M23K mutation perturbs transmission of conformational information, which modulates $\epsilon$ interactions with $F_0$ such that inhibition is not relieved and coupling efficiency is lowered. This is the primary effect of $\gamma$M23K on coupling.

The effect of $\gamma$M23K as well as other mutations in the $\gamma$- $\beta$ interface appear to perturb a balance of interactions between the subunits necessary for transmission of coupling information and energy. In addition, the $\gamma$M20K mutation appears to modulate the functional interaction of $\epsilon$ subunit with $F_0$. The effect of the structural perturbation on catalytic mechanism appears to be creation of a branched pathway that either bypasses or skips the coupling step. These results demonstrate the mechanistic linkage between catalysis and coupling that minimally involves $\beta$, $\gamma$, and $\epsilon$ subunits. Furthermore, we suggest that residues Met-23, Arg-242, $\beta^{340}$-DESEED 586, and $\epsilon$ residues (near $\epsilon$-Ser-108) form a common energy coupling domain that transmits conformational energy from $F_0$ to $F_1$ and vice versa at discrete point (times) within the turnover of the enzyme. These suggestions are currently being investigated by further experiments.

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