Effect of the ε-Subunit on Nucleotide Binding to Escherichia coli F₁-ATPase Catalytic Sites*

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F₁ is the catalytic sector of F₁F₀-ATP synthase, the enzyme responsible for ATP synthesis in the last step of oxidative phosphorylation. F₁ may be isolated in soluble form and is an active ATPase (“F₁-ATPase”) with a subunit stoichiometry of αβ₃γε. The three catalytic nucleotide-binding sites are located on the three ε-subunits (1–3). For Escherichia coli F₁, the minimal subunit composition able to achieve significant ATP hydrolysis rates is αβ₃γε. The small subunits ε and γ are required for functionally competent binding of F₁ to F₀ (4–7).

The ε-subunit is part of the central stalk connecting F₁ to F₀, the membrane sector of the ATP synthase complex. ε is tightly associated with the γ-subunit (8, 9). It appears to interact also with (one of) the ε subunits of F₀, and it can be cross-linked to the C-terminal domains of α and β (10–13). High-resolution structural models of isolated ε have been obtained based on NMR spectroscopy as well as x-ray crystallography (14–16).

In isolated F₁, the ε-subunit acts as an inhibitor. F₁ containing ε has ~10% of the ATPase activity of the ε-depleted form of the enzyme (5, 8, 17). How this inhibition is brought about is an interesting question, especially considering that ε is at least 35 Å away from the phosphate-binding pocket of the catalytic site. To gain more insight into the effects of ε on the function of F₁, we determined in this study the nucleotide binding affinities of the three catalytic sites in the absence and presence of the ε-subunit. The enzyme we used for this purpose was E. coli βγ331W mutant F₁. This enzyme, which shows normal functional properties, has a Trp genetically engineered into the adenine-binding domain of the catalytic site. The fluorescence response upon nucleotide binding makes this residue, β-Trp-331, a very sensitive probe for the occupancy of the catalytic site and allows the determination of thermodynamic and kinetic ligand binding parameters (18–20).

EXPERIMENTAL PROCEDURES

Protein Preparation and Analysis—Wild-type F₁ was from strain SWM1 (21), and βγ331W mutant F₁ was from strain SWM4 (18). Preparation of F₁ was as described (22). βγ331W mutant F₁ was depleted of the ε-subunit by immunoaffinity chromatography (23). Western blots (24) confirmed that the preparation was ~99% depleted of ε. The ε-subunit was prepared as described (25). The concentration of F₁ was determined using the Bio-Rad protein assay (26) with bovine serum albumin as the standard. The concentration of ε was measured by either the Bio-Rad protein assay or the method of Lowry et al. (27), both of which gave similar results.

Before use in ATPase and nucleotide binding assays, F₁ was transferred into a buffer containing 50 mM Tris/H₃O₂, pH 8.0, by passage through two consecutive 1-ml Sephadex G-50 centrifuge columns. ε-Depleted βγ331W mutant F₁ was generated by preincubating the ε-depleted enzyme (30–50 nM) for 15 min with a 4–5-fold concentration of the ε-subunit (120–200 nM). Dilution during the assays was <10%.

ATPase Activity Assay—The dependence of ATPase activity on the concentration of ε was determined after preincubation of F₁ (40 nM) with varied concentrations of ε in 50 mM Tris/H₃O₂, pH 8.0, for a minimum of 15 min at room temperature. The ATPase assay was started by adding 0.1 volume of a MgATP solution containing 50 mM Tris/H₃O₂, 40 mM MgSO₄, and 100 mM NaATP, pH 8.0. After a 1-min incubation at room temperature, the reaction was stopped by addition of SDS (5% final concentration). Released Pi was determined colorimetrically (28).

The dependence of ATPase activity on MgATP concentration (see Fig. 1) was measured in 50 mM Tris/H₃O₂, pH 8.0, at room temperature. F₁ concentrations were 30 nM in the presence of ε and 2.5 nM in its absence. The concentration of ε, when present, was 120 nM. The reaction was started by simultaneous addition of NaATP and MgSO₄ in a ratio of 2.5:1. Samples were withdrawn at 10- or 20-s intervals for a total time of 1 or 2 min. Released Pi was determined colorimetrically (29). Hydrolytic activities were determined from initial linear rates. MgATP concentrations were determined as described (30).

Fluorescence Measurements—All fluorescence measurements were performed in a buffer containing 50 mM Tris/H₃O₂, pH 8.0, at room temperature. The spectrofluorometers used were Spex Fluorolog 2, Amino-Bowman Series 2, and SLM 4800. The excitation wavelength was 295 nm. F₁ concentration was 30–50 nM. For MgATP titration, two different conditions were used. To make the results directly comparable to those obtained in the hydrolysis assay, NaATP and MgSO₄ were added in a constant ratio of 2.5:1 to a solution of protein in buffer (see Fig. 3). Alternatively, the cuvette contained protein in buffer plus 2.5 mM MgSO₄, and NaATP was added (see Fig. 5). In both cases, maximally two data points were acquired in a single experiment to avoid interference by the hydrolysis product MgADP. For MgADP titration,
The same result was obtained with nucleotide, the spectra of both enzyme forms were indistinguishable from the one for wild-type F1 shown in Fig. 2A. Catalytic Site Occupancy during Steady-state Hydrolysis in Absence and Presence of e-Subunit—Catalytic site occupancy in e-depleted and e-replete βY331W F1 is plotted in Fig. 3 as a function of substrate MgATP concentration. The conditions in this experiment were the same as those under which the hydrolysis data in Fig. 1 were obtained, i.e. ATP and MgCl₂ were present in a constant ratio of 2.5:1. Under these conditions, maximal hydrolysis rates are reached (32). In both cases, a binding model assuming three sites of different affinity gave a good fit, with \( K_{d1} = 0.02 \mu M, K_{d2} = 0.7 \mu M, k_{d3} = 21 \mu M \) for e-depleted βY331W F1 and \( k_{d1} < 0.01 \mu M, k_{d2} = 0.4 \mu M, k_{d3} = 21 \mu M \) for the e-replete enzyme. For both enzyme forms, fluorescence of the introduced β-Trp-331 residues is similar in each of the three catalytic sites.

RESULTS

Interaction of e-Depleted βY331W Mutant F1 with Isolated e-Subunit—Inhibition of βY331W mutant F1-ATPase activity by e was used as a signal to determine the affinity between the two proteins. e-Depleted βY331W F1 showed specific activities of 80–100 units/mg at 30 °C and 30–40 units/mg at 23 °C. Incubation of the e-depleted enzyme with a 10–20-fold concentration of e reduced activity by ~90%, to 5–7 units/mg at 30 °C and 3–4 units/mg at 23 °C. Titration of e-depleted F1 with e gave a curve indicating a \( K_d \) of ~1 nM (data not shown). Based on these results, we calculated that for the experiments described below, incubation of 30 nM e-depleted F1 with 120 nM e would give an enzyme that was ~99% e-replete.

Substrate Concentration Dependence of ATPase Activity of βY331W Mutant F1 in Absence and Presence of e—The ATPase activities of e-depleted and e-replete βY331W F1 as a function of MgATP concentration are shown in Fig. 1. Ignoring the ~10-fold difference in \( V_{max} \), the curves look remarkably similar. In both cases, the substrate concentration dependence followed simple Michaelis-Menten kinetics, with a single \( K_m \) value of 27 μM in the absence of e and 21 μM in its presence.

Fluorescence Properties of βY331W Mutant F1 in Absence and Presence of e—To determine the affinity between e and βY331W F1, there is a Trp fluorescence maximum at 345 nm, and the e-replete enzyme has one at 339 nm; both spectra are red-shifted as compared with wild-type F1 (Fig. 2A). e itself contains no Trp and has no significant Trp fluorescence (Fig. 2A). As noted previously (11), saturation with nucleotide virtually completely quenches the fluorescence of the three introduced β-Trp-331 residues in βY331W F1 prepared by standard procedures. The same result was obtained here with both e-depleted and e-replete βY331W F1. After saturation with nucleotide, the spectra of both enzyme forms were indistinguishable from the one for wild-type F1 shown in Fig. 2A, indicating that the spectral differences between e-depleted and e-replete βY331W F1 are due to an effect of e on the fluorescence of the introduced β-Trp-331 residues. Fig. 2B shows the Trp fluorescence spectra of β-Trp-331 in the absence and presence of e. In the absence and presence of e, the maxima are at 345 and 350 nm, respectively. Thus, in an empty catalytic site, the environment of β-Trp-331 is highly polar, especially in the e-containing enzyme. Analysis of spectra generated in an MgADP binding experiment with both types of enzyme showed no significant shift of the respective β-Trp-331 spectrum during the titration (data not shown). Thus, the influence of e on β-Trp-331 fluorescence is similar in each of the three catalytic sites.

By 31% quenching (by 16%) of the Trp fluorescence of the γ-subunit by e was reported. The apparent absence of influence of e on the fluorescence of wild-type F1 suggested by the data reported here indicates that either (a) the Trp residue(s) in γ that responds to e does not contribute significantly to the fluorescence of F1, or (b) once γ is incorporated into F1, its fluorescence is no longer affected by e.
The data from Figs. 1 and 3. Of course, the occupancy data on the catalytic sites occupied, using both curves clearly provide very good fits to the actual behavior of hydrolysis rates (i.e., the catalysis rate of enzyme molecules with only one or two sites filled is zero). These theoretical curves provide very good fits to the measured data points assuming a model with three binding sites of different affinity. Further details are given under “Experimental Procedures.”

Fig. 4 (symbols) shows relative specific ATPase activity plotted as a function of the fraction of catalytic sites occupied, using the data from Figs. 1 and 3. Of course, the occupancy data on the catalytic sites are the average over all enzyme molecules. The lines are theoretical curves assuming that only enzyme molecules with all three catalytic sites filled achieve measurable rates. A fit of theoretical curves to the measured data points assuming a non-zero activity for enzyme molecules with two filled catalytic sites resulted in nearly identical curves, with a “bi-site” activity of 0% of the catalytic rate of enzyme molecules with all three catalytic sites filled have ATPase activity (equation given in Ref. 18).

Effect of e-Subunit on Nucleotide Binding Affinities—As described above, e appears to have little effect on the MgATP affinities of catalytic sites 2 and 3. Under the conditions used in Fig. 3 (a constant ATP/Mg$^{2+}$ ratio of 2.5:1 to achieve maximal activity), the exact calculation of submicromolar MgATP concentrations is difficult because of uncertainty in assessing the true Mg$^{2+}$ concentration and dependence on the value of $K_d$ for the Mg$^{2+}$-APc complex. To overcome this, we repeated the MgATP binding experiments using a constant 2.5 mM Mg$^{2+}$ concentration, where virtually all ATP is in the metal-complexed form. Fig. 5 shows that at low MgATP concentrations, the e-replete $K_{d1}$ values derived from Fig. 5 indicate that $e$ is higher for MgADP than for MgATP. The $K_{d1}$ value of 0.06 M was derived in the absence of $e$, whereas there is only a small effect on site 2 ($K_{d1}$ = 2.7 ± 1.0 M, respectively) and no effect on site 3 ($K_{d1} = 0.02$ M in both cases).

The influence of the $e$-subunit on the affinities for MgADP is shown in Fig. 6. Again, at low ligand concentrations, the e-replete enzyme has a higher affinity for MgADP than the e-depleted form. The $K_{d1}$ values for the e-replete enzyme were $K_{d1} = 0.06$ M and $K_{d2} = 19$ M, with 1.0 sites of class 1 and 1.7 sites of class 2. The respective values for the e-depleted $F_1$ were $K_{d1} = 0.7$ M (0.9 sites) and $K_{d2} = 25$ M (1.9 sites). Thus, $e$ increases the MgADP binding affinity of catalytic site 1 by ~10-fold, whereas the affinities of sites 2 and 3 are not significantly affected.

No significant effect of $e$ on the binding affinities for free ATP and ADP was detected. In all cases, a model with a single type of binding site gave a satisfactory fit. For ATP, $K_a$ was 41 M (2.7 sites) in the absence of $e$ and 53 M (2.8 sites) in its presence. For ADP, the respective values were 63 M (2.5 sites) and 85 M (2.9 sites).

**DISCUSSION**

General—The goal of this study was to investigate the influence of $e$ on the nucleotide binding affinities of the catalytic sites. The data from Figs. 1 and 3. Of course, the occupancy data on the catalytic sites occupied, using both curves clearly provide very good fits to the actual behavior of hydrolysis rates (i.e., the catalysis rate of enzyme molecules with only one or two sites filled is zero). These theoretical curves provide very good fits to the measured data points assuming a model with three binding sites of different affinity. Further details are given under “Experimental Procedures.”

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sites of F$_1$-ATPase, using a specific fluorescence probe for catalytic site occupancy, β-Trp-331. Previously, we had shown that the βY331W mutation did not affect the enzymatic properties; βY331W F$_1$ and F$_2$F$_3$ are fully competent in ATP hydrolysis and synthesis (18, 20). Here we first demonstrated that the interaction between βY331W F$_1$ and the ε-subunit, with $K_d = 1$ nM, is very similar to that found in the wild-type enzyme (8, 33) and that, as in wild-type F$_1$ (8), ε reduced the ATPase activity of βY331W F$_1$ by ~90%.

Effect of ε on Catalytic Site Nucleotide Binding—ε did not affect the binding of free ATP or ADP to any of the catalytic sites. For magnesium-nucleotide binding, the only pronounced effect was at the high-affinity catalytic site 1, where the affinity for MgADP and MgATP was ~10-fold higher in the ε-depleted than in the ε-depleted form of the enzyme (Figs. 5 and 6). One reason for this difference could be a reduced off-rate for the magnesium-nucleotide complex in the presence of ε. Interestingly, previous work from one of our laboratories (34) showed that release of product Pi from ε-depleted F$_1$ in “uni-site catalysis”, i.e., under conditions where only the high-affinity site is filled with substrate, was reduced by a similar factor compared with that from the ε-depleted enzyme. The results presented here support the conclusion that the ε-subunit decelerates a conformational change step that is necessary to release ligands from the high-affinity binding site.

Further Evidence That MgATP Binding at Site 3 Determines Overall Rate of Hydrolysis—In ε-depleted and ε-replete βY331W F$_1$, a single $K_m$ value of 20–30 μM was sufficient to describe the substrate concentration dependence of enzymatic activity (Fig. 1), and in both cases, $K_m$ clearly corresponded to $K_d$, i.e., the $K_d$ for MgATP at the low-affinity site 3. A re-plot of the data (Fig. 4) confirmed that in both ε-depleted and ε-replete βY331W F$_1$, only enzyme molecules that have all three catalytic sites filled contribute significantly to the measured hydrolytic activity (“tri-site catalysis”).

In earlier reports using wild-type E. coli F$_1$, a second, lower $K_m$ was found (34, 35), which was ascribed to turnover due to enzyme molecules with two filled catalytic sites. However, neither the higher nor the lower $K_m$ was significantly affected by the presence or absence of ε (34), consistent with the findings here that $K_{d1}$ and $K_{d2}$ for MgATP are similar in the ε-depleted and ε-replete enzymes. The fact that substantial bi-site activity was not detected in the current work or in a previous study (18) is likely due to differences in experimental protocol. Bi-site catalysis was evident when we investigated the hydrolysis of MgTNP-ATP by βY331W F$_1$. For this substrate, two $K_m$ values were required to describe the enzymatic activity adequately, and the lower $K_m$ corresponded extremely well to $K_{d2}$ (36). What should be emphasized, however, is that even in the cases where bi-site activity is described, tri-site activity is clearly the physiologically relevant working mode of the enzyme in the ε-replete or ε-depleted enzyme.

Relevance of Current Studies to Inhibitory Properties of ε—It is intriguing that the ε-induced increase in affinity and the reduction of the product release rate from the high-affinity site are by a factor (~10-fold) similar to that for the decrease in the multi-site hydrolysis rate in the presence of ε, suggesting that the two events might be closely connected. According to the model for multi-site hydrolysis recently proposed by two of us (3, 37), MgADP release from the low-affinity site 3 is rate-limited. The fact that the ε-subunit influences the fluorescence spectrum of the β-Trp-331 residues in all three catalytic sites in a similar manner (Fig. 2) shows that the long-range interactions between ε and the catalytic sites are not restricted to any single site, but are of a more global nature. Also, it may be noted in this context that according to Gruber and Capaldi (38), the β-subunit to which ε can be cross-linked is neither the one that carries catalytic site 1 nor the one that carries site 3, but the one containing the medium-affinity site 2. Thus, ε may affect product release from the low-affinity site 3 by a mechanism similar to that responsible for reduction of uni-site product release. As $K_{d3}(\text{MgADP})$ is essentially identical in the absence and presence of ε, from $K_d = K_{d3}/K_{on}$ it follows that a 10-fold increase in the product off-rate in the absence of ε would have to be accompanied by a similar increase in the on-rate. Such parallel changes in kinetic constants are not unprecedented in F$_1$, $K_{d1}$ for binding of MgADP and MgAMP-PNP is ~0.1 μM; however, $K_{on}$ (and consequently, $k_{cat}$) for MgAMP-PNP is ~2 orders of magnitude less that that for MgADP (18). Further experiments will be necessary to determine the kinetics of each of the individual reaction steps in multi-site catalysis and the detailed inhibitory mechanism of ε.

Heterogeneity of Preparations with Respect to ε Content Does Not Affect Conclusions Regarding Overall Mechanism—Finally, it is worthwhile to reexamine previous experimental data obtained with βY331W F$_1$ (reviewed in Ref. 3) in light of the results of the present study. As judged from the specific activities (5.9 units/mg at 23 °C and 13 units/mg at 30 °C) (18), from the MgATP and MgADP binding affinities at site 1 (0.028 and 0.08 μM at 2.5 mM Mg$^{2+}$) (39, 40), as well as from the wavelength position of the β-Trp-331 fluorescence spectrum ($\lambda_{max} = 349$ nm) (18), the enzyme prepared by the standard procedure in our laboratory is largely (>90%) ε-replete, and due to the relatively high concentration of enzyme used (~50 nm), it remains ε-replete in both hydrolysis and nucleotide binding assays. On the other hand, it appears that some degree of ε-depletion can occur as a result of the nucleotide depletion procedure involving Sephadex G-50 gel filtration in 50% (v/v) glycerol-containing buffer. Such nucleotide-depleted βY331W F$_1$ has a specific activity of 9.4 units/mg (at room temperature) and $K_{d3}(\text{MgADP}) = 0.14$ μM (18), indicating that up to 20% of the enzyme molecules may not contain ε.

However, in general, such partial ε-depletion does not appear to present a problem in experiments studying multi-site hydrolysis, as the results described here show that the mechanism of the ε-depleted and ε-replete enzymes is fundamentally the same. Thus, recent criticism (41) of the Trp fluorescence approach to correlate hydrolysis and nucleotide binding data is

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*The abbreviations used are: MgTNP-ATP, Mg-2'(3')-O-(2,4,6-trinitrophenyl)adenosine 5'-triphosphate; MgAMP-PNP, Mg-5'-adenylylimidodiphosphate.*
unjustified. Of wider relevance, previous reports in the literature using *E. coli* F₁ where mixtures of e-depleted and e-replete enzyme molecules might have occurred can nevertheless be interpreted with confidence.

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3 It should also be emphasized that our hydrolysis experiments are designed to minimize concentrations of free Mg^{2+}; thus, no inhibition by Mg^{2+} or MgADP is expected under these conditions (see, for example, Ref. 42).