Unisite $[\gamma^{-32}\text{P}]{\text{ATP}}$ hydrolysis was studied in ECF$_1$ from the mutant $\beta\text{E381C}$ after generating a single disulfide bond between $\beta$ and $\gamma$ subunits to prevent the rotation of the $\gamma/e$ domain. The single $\beta$-$\gamma$ cross-link was obtained by removal of the $\delta$ subunit from F$_1$, and then treating with CuCl$_2$ as described previously (Aggeler, R., Haughton, M. A., and Capaldi, R. A. (1996) J. Biol. Chem. 270, 9185–9191). The mutant enzyme, $\text{E381C}$, had an increased overall rate of unisite hydrolysis of $[\gamma^{-32}\text{P}]{\text{ATP}}$ compared with the wild type ECF$_1$, due to increases in the rate of ATP binding ($k_{-1}$), P$_i$ release ($k_{+g}$), and ADP release ($k_{+s4}$). Release of bound substrate ($[\gamma^{-32}\text{P}]{\text{ATP}}$) was also increased in the $\text{E381C}$ mutant. Cross-linking between Cys-381 and the intrinsic Cys-87 of $\gamma$ caused a further increase in the rate of unisite catalysis, mainly by additional effects on nucleotide binding in the high affinity catalytic site ($k_{-1}$ and $k_{+s4}$). In $\delta$-subunit-free ECF$_1$ from wild type or $\beta\text{E381C}$ F$_1$, addition of an excess of ATP accelerated unisite catalysis. After cross-linking, unisite catalysis of $\text{E381C}$ was not enhanced by the cold chase. The covalent linkage of $\gamma$ to $\beta$ increased the rate of unisite catalysis to that obtained by cold chase of ATP of the noncross-linked enzyme. It is concluded that the conversion of Glu-381 of $\gamma$ to Cys induces an activated conformation of the high affinity catalytic site with low affinity for substrate and products. This state is stabilized by cross-linking the Cys at $\beta$381 to Cys-87 of $\gamma$. We infer from the data that rotation of the $\gamma/e$ rotor in ECF$_1$ is not linked to unisite hydrolysis of ATP at the high affinity catalytic site but to ATP binding to a second or third catalytic site on the enzyme.

F$_1$F$_0$-type ATPases are membranous protein complexes responsible for oxidative and photosynthetic ATP synthesis in eubacteria, mitochondria, and chloroplasts. The simplest form of this enzyme, as found in Escherichia coli (ECF$_1$, F$_1$), contains eight different subunits. Five of these subunits ($\delta$, $\beta_3$, $\gamma_1$, $\delta_1$, $\epsilon_1$) are located outside the membrane in the catalytic $F_1$ portion. The other three subunits ($\alpha_1$, $\beta_2$, $\epsilon_9$–$12$) form a channel that transports protons through the membrane (for recent reviews, see Refs. 1 and 2). Each enzyme complex contains three catalytic sites that are characterized by high, medium, and low affinities for nucleotides (1–2, 15–16), energization (4–6) and that they shift their positions between the $\alpha$ and $\beta$ subunits of soluble F$_1$, in response to nucleotide binding (7–9). Recently, it has been established conclusively that these movements represent rotation of a domain formed by the $\gamma$ and $\epsilon$ subunits (10, 11). Such rotation has been visualized directly during ATP hydrolysis in the soluble $F_1$ (12, 13) and is inferred by cross-linking studies of the hydrolytic and synthetic reaction when catalyzed by the whole $F_1F_0$ complex (11, 14).

As yet, no correlation has been established between the rate of rotation of the $\gamma/e$ domain and the kinetics of individual steps in the ATP hydrolysis or synthesis reactions. However, the hydrolytic activity of the enzyme is almost fully inhibited by cross-linking either $\gamma$ or $\epsilon$, or both subunits, to $\alpha$ or $\beta$ subunits through engineered disulfide bonds (8–10), thereby confirming the direct linkage between catalytic site events and rotation. The driving force for this rotation in the direction of ATP hydrolysis could be the binding energy of ATP and/or the free energy change associated with the ATP splitting reaction. However, complicating any analysis, ATP can bind in three catalytic sites that are characterized by high, medium, and low affinities for nucleotides (1–2, 15–16), a priori, binding in any of these sites could drive the rotation.

To some extent, the functioning in these sites can be differentiated because ATP hydrolysis in the high affinity catalytic site can be monitored by so-called “unisite” catalysis measurements (17). It is distinguished from nucleotide binding into low affinity sites which accelerates product release from the first site, as in cold chase experiments (18). Unisite catalysis is measured with substoichiometric amounts of $[\gamma^{-32}\text{P}]{\text{ATP}}$ in relation to $F_1$ and is characterized by a highly exergonic ATP binding step ($K_g$), a reversible ATP hydrolysis/synthesis equilibrium that occurs with a negligible change in free energy ($K_i$), and a very slow product release step which is rate-limiting ($k_{-3}$ and $k_{-s4}$) (see Equation 1 and review in Ref. 19). Therefore, if subunit rotation is coupled to unisite catalysis, ATP binding in the high affinity site would be the most probable driving force (20). The kinetic steps of the unisite catalytic cycle are as follows.

$$
F_1 + \text{ATP} \overset{k_{-1}}{\rightleftharpoons} F_1 \cdot \text{ATP} \overset{k_{+s4}}{\rightleftharpoons} F_1 \cdot \text{ADP} \cdot P_i \overset{k_{+s3}}{\rightleftharpoons} F_1 \cdot \text{ADP} + P_i \overset{k_{+s2}}{\rightleftharpoons} F_1 + \text{ADP}
$$

(Ref. 1)

Here, we measure unisite catalysis in ECF$_1$ in which the $\gamma$ subunit has been cross-linked to a $\beta$ subunit to prevent rota-
tion. Our results are interpreted to indicate that ATP binding at a catalytic site other than the high affinity site, drives the rotation of the γ and ε domain.

EXPERIMENTAL PROCEDURES

Materials—[γ-32P]ATP (PB 218) was purchased from Amersham Pharmacia Biotech, and [α-32P]ATP was a gift from the laboratory of Dr. Peter von Hippel of this Institute of Molecular Biology. ATP was from Sigma. The mutant βE381C is described elsewhere (21). Wild type and mutant F1-ATPases were purified as described before (22). EC1, was depleted of δ subunit by gel filtration chromatography through Sephacryl S-300 in the presence of 0.5% LDAO. Enzyme (8–10 mg) was dissolved in 1 ml of a buffer containing 50 mM Tris, 20% glycerol, 2 mM EDTA, 1 mM ATP, 1 mM DTT, and 40 mM EACA, pH 7.4 (4 °C), along with 0.5% LDAO (buffer A). Samples were loaded onto a Sephacryl column (1.2 m x 1.0 cm), and protein was eluted with the same buffer. Samples lacking the δ subunit based on SDS-polyacrylamide electrophoresis were collected and concentrated to 2–3 mg/ml in Amicon tubes and stored in liquid nitrogen.

CuCl2-induced Cross-linking of F1, Preparations—Cross-linking was induced by CuCl2 as described previously (21). Before starting the cross-linking reactions, enzyme dissolved in buffer A under liquid nitrogen was defrosted at room temperature and precipitated with 70% saturation of ammonium sulfate, was used at a concentration of 0.2 mg/ml. CuCl2 (10 mM glucose. The hexokinase reaction was allowed to proceed for

Unisite Catalysis without Rotation of the γ-ε Domain

Unisite reactions were started by adding 50 µl of [γ-32P]ATP to 50 µl of F1 during rapid mixing in a 13 x 100 mm glass test tube. The [γ-32P]ATP/F1 ratio used was 0.1 or 0.2 without significant differences on the results obtained in both conditions. The final concentration of F1 was 0.5 µM and that of [γ-32P]ATP was 0.1 or 0.05 µM. Reactions were started by mixing at different reaction times with 0.4 ml of trichloroacetic acid (60% final concentration). In the case of cold chase experiments, 0.1 µl of 10 mM MgATP was added to the F1 + [γ-32P]ATP mixture at the times shown, and the reactions were stopped 2 s later with acid. In the control samples, 5 mM DTT was present at the times shown, and the reactions were stopped with 1.3 N HCl. The remaining [32P]ATP was hydrolyzed by boiling for 12 min, and the [32P]Pi, produced was extracted as described above. The radioactivity of the aqueous phase was taken as the amount of [32P]glucose-6-phosphate formed, i.e., [γ-32P]ATP that was not bound to F1. Controls showed that less than 1% of the [32P]glucose-6-phosphate was heat-resistant, and that the efficiency of the hexokinase trap mixed with F1 was 97–99%. The decay on the hexokinase accessible [γ-32P]ATP against time was used to calculate the rate constant of [γ-32P]ATP binding, following a second order association process according to Penefsky (27). The further mathematical treatment of the rate constant [γ-32P]ATP/F1 release (28) showed that the amount of [γ-32P]ATP released in the first 10 s of reaction was negligible, allowing for a good estimation of k-2. On the other hand, k-1 was measured by a similar hexokinase trap according to the method of Penefsky (27), in which the [γ-32P]ATP/F1 mixture was aged for 30 s and the free 32P was removed by gel filtration through Sephadex columns equilibrated with the standard unisite buffer containing 1 mg/ml bovine albumin (BSA). After saving aliquots for determination of [γ-32P]ATP and [32P]Pi, coeluted with F1, the eluent was diluted 10-fold in the same buffer containing 1 mg/ml BSA, 1 mg/ml hexokinase, and 20 mM glucose. At different times, aliquots were removed and the hexokinase reactions were stopped with 1.3 N HCl. The amount of [32P]glucose-6-phosphate formation, measured as described above, was used to calculate ECF1 from the mutant E381C was used in the present study. The properties of this mutant have been described in detail elsewhere (21). Cross-linking in essentially full yield can

RESULTS

ECF1, from the mutant βE381C was used in the present study. The properties of this mutant have been described in detail elsewhere (21). Cross-linking in essentially full yield can
Unisite Catalysis without Rotation of the $\gamma$-$\epsilon$ Domain

The removal of the $\delta$ subunit had very little effect in the rate of acid quench-measured unisite hydrolysis of $[\gamma^{32}\text{P}]$ATP in either wild type or mutant-(bE381C) ECF1, as reported previously (35) and see Table I. Fig. 2 compares the rates of unisite catalysis of wild type ECF1 and bE381C, both before and after cross-linking. The rates of unisite catalysis by wild type ECF1 are comparable with those obtained by others under similar experimental conditions (35, 40); see Table I for rate constants of catalysis. As evident in Fig. 1, the substitution of Cys for Glu-381 in the mutant bE381C significantly increased the rate of unisite ATP hydrolysis of ECF1, and this rate is increased more dramatically after cross-linking of the Cys at position 381 of $\beta$ with Cys-87 of the $\gamma$ subunit.

Before making a detailed kinetic analysis of the unisite activity of the bE381C mutant, the possible contribution of multisite activity in the present experiments was examined by testing the effect of sodium azide (NaN$_3$). NaN$_3$ is an inhibitor which effectively blocks multisite ATPase activity of F$_1$-ATPases without significant effect on unisite catalysis, presumably by affecting catalytic site cooperativity (36, 37). Previous studies had suggested that cooperative or multisite catalysis could contribute even when ratios of $[\gamma^{32}\text{P}]$ATP to F$_1$, of 0.3 mol/mol were used (38). Although cross-linking itself inhibits the multisite catalysis, this control is still relevant because small amounts of non-cross-linked enzyme are present and could be providing all of the $[\gamma^{32}\text{P}]$ATP hydrolysis being observed. However, as shown in Fig. 2, NaN$_3$ at concentrations that completely blocked multisite catalysis in noncross-linked enzyme (results not shown; Refs. 36 and 37) had no effect on the rates of unisite catalysis after cross-linking. Taken together, the above results confirm that the ATP hydrolysis being followed occurs in the so-called “high affinity” catalytic site in the cross-linked enzyme.

Table I lists the rate and equilibrium constants that were measured. In relation to wild type ECF1, the rate of ATP binding ($k_{+1}$) is faster for the bE381C mutant both before and after cross-linking. Also, $[\gamma^{32}\text{P}]$ATP release was faster in the mutant by a factor of 25 before cross-linking and more than 100-fold after cross-linking. The result is a 10-fold decrease in the affinity for ATP. As indicated in Table I, the mutant showed a more than 20-fold increase in $k_{-2}$, the rate of P$_i$ release, which was not enhanced greatly by cross-linking. The cross-linking also increased the rate of ADP release from the mutant ECF1. Table I also lists $K_m$ for the different preparations. This equilibrium constant was not greatly affected in the mutant with or without cross-linking.

The effect of a cold chase of excess ATP on unisite catalysis for the wild type and mutant is shown in Fig. 3. There was the expected burst of $[\gamma^{32}\text{P}]$ATP hydrolysis after addition of cold ATP in the wild type ECF1, and in enzyme from the mutant without cross-linking. However, after cross-linking, the rate of unisite catalysis carried out by the bE381C mutant was by itself as fast as the accelerated unisite catalysis of the non-cross-linked mutant. Unisite catalysis of the bE381C mutant was also much faster than the promoted and non-promoted unisite catalysis carried out by the wild type enzyme, which is limited by the rate of $[\gamma^{32}\text{P}]$ATP binding (Fig. 3).

**DISCUSSION**

In the mutant bE381C, a cross-link can be obtained between $\beta$ and $\gamma$ subunits in essentially full yield. Previous studies made in ECF1 from the triple mutant bE331W:bE381C:S108C (39) have established that binding of ATP to this cross-linked F$_1$ is similar to that of non-cross-linked enzyme, i.e. there is one high affinity binding site ($K_{d1} = 90–200$ nM), a loose site ($K_{d2} = 2–7$ $\mu$M), and a weak binding site ($K_{d3} = 40–50$ $\mu$M). The results here show that enzyme cross-linked between $\beta$ (at Cys-381) and $\gamma$ subunit have a 20-fold increase in $k_{+1}$, and a 10-fold decrease in $K_m$ for ATP.

![Fig. 1. SDS gel electrophoresis of the bE381C mutant before and after cross-linking induced by CuCl$_2$.](image-url)
Rate constants were measured according to those under “Experimental Procedures.” Experiments were made with [γ-32P]ATP/ECF1 ratios of 0.1 and 0.2. The wild type values were measured in duplicate experiments and those for the βE381C ECF, in three or four different determinations. Standard deviations were omitted for simplicity, but these were no higher than 20%. Significant changes produced by the βE381C mutation are highlighted, and further increases induced by cross-linking are highlighted in italics.

|                | \(k_{-1}\) | \(k_{-1}\) | \(K_{1}\) | \(K_{2}\) | \(k_{+1}\) | \(k_{+1}\) |
|----------------|------------|------------|----------|----------|-----------|-----------|
| \(\text{wtECF}_1\) (+β) \(m\) | 1.1 | 2.5 | 4.4 | 2.9 | 12.0 | 1.6 |
| \(\text{wtECF}_1\) (+β, this work) | 1.2 | 2.2 | 5.4 | 0.18 | 7.5 | — |
| \(\text{wtECF}_2\) (+β, this work) | 0.55 | 2.5 | 2.2 | 1.4 | 12.0 | — |
| \(\beta\text{E381C ECF}_1\) (–δ, DT) | 0.77 | 2.2 | 3.5 | 2.1 | 10.0 | — |
| \(\beta\text{E381C ECF}_1\) (–δ, β-γ) | 5.3 | 60.0 | 0.88 | 1.0 | 220.0 | 2.2 |
| \(\beta\text{E381C ECF}_1\) (–δ, β-γ) | 6.1 | 310.0 | 0.20 | 2.1 | 280.0 | 7.7 |

\(a\) Values taken from Ref. 40.
\(b\) Values taken from Ref. 35.

**Fig. 2.** Effect of CaCl₂ induced cross-linking on the unisite catalysis of βE381C ECF₁, after removal of the δ subunit. Unisite hydrolysis of 0.05 μM [γ-32P]ATP by 0.5 μM ECF₁ was measured as explained under “Experimental Procedures.” The circles show the amount of [γ-32P]ATP hydrolyzed by the βE381C F₁ in the presence (○—○) and in the absence (○—○) of 5 mM DT. For comparison, the same experiment was made with the wild type ECF₁ (×—×). For the reactions measured in the presence of NaN₃ (Δ—Δ), ECF₁ was preincubated with this inhibitor for 10 min before starting the experiment. The experimental points are the average of triplicate measurements. All reactions were stopped by an acid quench at the times shown by the experimental points.

**Fig. 3.** Acceleration of unisite catalysis in β-γ cross-linked βE381C ECF₁ (–δ). The unisite catalysis of the βE381C F₁(–δ) was measured at a [γ-32P]ATP/ECF₁ ratio of 0.1. In the presence of 5 mM DT, nonpromoted unisite catalysis (○—○) was stopped by the standard acid quench. Promoted unisite catalysis (○—○) was measured by adding a volume of 10 μl MgATP to ECF₁, undergoing unisite catalysis and allowing the reaction to proceed 2 s further during rapid mixing before adding the acid. The open circles indicate the time at which the reactions were stopped. The same procedure was used to measure the promoted (Δ—Δ) and non-promoted (×—×) unisite catalysis of the βE381C F₁(–δ) previously cross-linked between β and γ subunits. For comparison, the figure also shows the promoted (●—●) and non-promoted (×—×) unisite catalytic activity of wild type ECF₁ (–δ). For the wild type ECF₁, promoted experimental data are plotted at the time of addition of the cold chase, but the reactions were stopped 1 min later, the same results were obtained if the cold chase reactions of the wild type ECF₁ were stopped after 2 or 5 s. Bound [γ-32P]ATP (○—○) was also measured in parallel for the wild type enzyme by the hexokinase trap method.
similar and direct effect on catalysis in the highest affinity site, as would be expected if γ subunit binding is determined and/or is a determinant of nucleotide binding affinities. Cross-linking between β and γ subunits of the mutant βE381C which inhibits multisite catalysis by 97%, was found to further increase the rate of unisite catalysis to the point that the reaction was essentially over within the first few seconds under the standard conditions used here. Curve fitting was used to analyze the kinetics of unisite catalysis as described under “Experimental Procedures.” Good fits (S.E. > 20% in the constants fitted) were obtained for wild type enzyme and for mutant, giving values of 0.71 and 0.33 (wt) and 0.84 and 0.84 (βE381C) respectively for $k_{-2}$ and $k_{+2}$. These values are similar to those reported earlier for wild type enzymes (35, 40) but lower than those of the γT106 mutation (6, 29), presumably due to the differences introduced by this mutation and/or its labeling with a fluorescent probe. The kinetics of unisite catalysis after cross-linking of βE381C was so fast that no satisfactory fit could be obtained (S.E. ≥ 50% in $k_{-2}$ and $k_{+2}$), therefore it remains unclear if cross-linking alters these two rate constants.

As shown in Fig. 3, the effect of covalently linking βCys-381 to γCys-87 is to increase the rate of unisite catalysis to that of non-cross-linked enzyme obtained with a cold chase of ATP. After cross-linking, the ATP cold chase did not increase the observed rate of unisite catalysis. The implication is that ECF1 from the mutant βE381C is in a conformation facilitating the rapid release of nucleotide and P$_i$ that occurs on substrate binding in a second or third site. This conformation is then stabilized by cross-linking. A similar activation of the high affinity catalytic site can be obtained in Rhodospirillum rubrum F$_1$-ATPase in the presence of Ca$^{2+}$, but in this case, multisite ATPase activity is also enhanced (45). Further studies are needed to determine if these activated states are structurally similar.

The rapid rate of bond cleavage of ATP to ADP + P$_i$ in enzyme in which the γ subunit is covalently linked to a β subunit indicates that rotation of the γ/ε subunit domain is not driven by, and does not require, nucleotide binding, or bond cleavage, in the high affinity catalytic site. Additional testing requires that methods such as those employed by Sabbett et al. (12) or Noji et al. (13) for demonstrating rotation of the γ subunit are adapted to unisite conditions. An absence of rotation of the γ/ε subunits driven by ATP binding or bond cleavage in the high affinity catalytic site does not necessarily rule out that these reactions drive the coupling of ATP hydrolysis (or synthesis) to proton translocation. Conformational changes induced by ATP hydrolysis within the high affinity catalytic site could be transmitted to the ε subunit and result in translocation of protons without rotation of the γε/εc subunit ring domain (presently, the favored model of energy transduction within the ATP synthase (10, 44, 46)). Instead, rotation could realign the molecule after each catalytic site-driven proton translocation event for the next turnover to proceed. In this connection, it is interesting that conformational changes have been observed by fluorescent probes attached at positions 8 and 106 of the γ subunit which correlate with unisite catalysis (6, 29). Critical testing of the rotation of the ε subunit as a function of catalytic site events remains an important prerequisite to getting a better understanding of the mechanism of the ATP synthase.

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