The Stalk Region of the *Escherichia coli* ATP Synthase

TYROSINE 205 OF THE γ SUBUNIT IS IN THE INTERFACE BETWEEN THE F₁ AND F₀ PARTS AND CAN INTERACT WITH BOTH THE ε AND ε OLIGOMER*

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The soluble portion of the *Escherichia coli* F₁F₀ ATP synthase (ECF₁) and *E. coli* F₁F₀ ATP synthase (ECF,F₀) have been isolated from a novel mutant γY205C. ECF₁ isolated from this mutant had an ATPase activity 3.5-fold higher than that of wild-type enzyme and could be activated further by maleimide modification of the introduced cysteine. This effect was not seen in ECF₁F₀. The containing the mutation γY205C was bound to the membrane-bound portion of the *E. coli* F₁F₀ ATP synthase (ECF₁F₀) isolated from mutants cA39C, cQ42C, cF43C, or 44 in the c subunit but not at position 39. Using Cu²⁺ treatment or reaction with 5,5'-dithio-bis(2-nitro-benzoic acid) induced disulfide bond formation between the Cys at position 205 and a Cys residue at positions 42, 43, or 44 in the ε subunit but not at position 39. Using Cu²⁺ treatment, this covalent cross-linking was observed in high yield by Cu²⁺. No cross-linking was observed in hybrid enzymes in which the Cys was at position 10, 65, or 108 of the ε subunit. Cross-linking of γ to ε had only a minimal effect on ATP hydrolysis. The reactivity of the Cys at position 205 showed a nucleotide dependence of reactivity to maleimides in both ECF₁ and ECF₁F₀, which was lost in ECF₁ when the ε subunit was removed. Our results show that there is close interaction of the γ and ε subunits for the full-length of the stalk region in ECF₁F₀. We argue that this interaction controls the coupling between nucleotide binding sites and the proton channel in ECF₁F₀.

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To explore the interactions of the region of the γ subunit between residues 202 and 230 in more detail, we have now converted γ Tyr-202 to a Cys. This introduced Cys is shown to cross-link by disulfide bond formation with Cys residues in both the ε subunit and in the polar loop of the ε subunits. Functional consequences of such cross-links are described.

EXPERIMENTAL PROCEDURES

Strains and Plasmid Construction—The M13mp18 template used consisted of a 1.4-kilobase ung-containing Smal-EcoRI fragment as described by Aggerl and Capaldi (20). The oligonucleotide used for the mutagenesis was ATAAAAATCTGGATTGCTTCGTACGACCGATC, where the first underlined G changes a TAC codon (Tyr) to TGC (Cys), creating the γY205C mutation, and the CTC codon replaces CTC, retaining Leu at position γ206. A Real site is eliminated at position 893 of the unc operon (numbering according to Walker et al. (21)), which is diagnostic of the presence of the mutation. The mutant phage was identified by Real digestion and transferred into pRA100 (14). The final plasmid containing the mutation γY205C was called pCT100. Mutants cA39C, cQ42C, cP43C, and cD44C were obtained from Dr. Robert Fillinger and are described elsewhere (18). For construction of the double mutant γY205C/cQ42C, plasmids pYZ203 and pYZ217 were isolated as described elsewhere (13, 14, 16). For construction of the double mutant γY205C/cQ42C, plasmids pYZ203 and pYZ217 were isolated from the strains expressing mutants cA39C and cQ42C, respectively. The 5.8-kilobase XhoI/SsrI fragment of plasmid pCT100 containing the unc gene with the γY205C mutation was used to replace the corresponding region of plasmids pYZ203 and pYZ217. These new plasmids are called pSW101 and pSW102. Strain AN1460 (uncB, uncD, argF, enaA, sacA14) (14) was transformed with plasmids pCT100, pSW101, pSW102, and pYZ217 for expression of mutant enzyme. ε subunit mutants were obtained, and the ε subunit was isolated as described elsewhere (13, 14, 16).

Labeling ECF1 and ECF1F0 ATPases with Maleimides—ECF1 and ECF1F0, ATPases with Maleimides—ECF1, and ECF1F0, were isolated from various mutants and from AN1460 as a control for biochemical studies, as described in Wise et al. (22) and Aggerl et al. (23), respectively. For reaction of ECF1 with maleimides, enzyme was passed through two consecutive centrifuging columns (Sephadex G-50, fine, 0.5 × 6 cm equilibrated with 50 mM MOPS, pH 7.0, 0.5 mM EDTA, 10% glycerol). ECF1 was then labeled at a concentration of 1.0–2.0 mg/ml with 200 μM ATP or 200 μM AMP-PNP in column buffer at room temperature. In each experiment, one sample of enzyme was reacted with 2 mM ATP or 2 mM AMP-PNP in column buffer at room temperature. In each experiment, one sample of enzyme was incubated in Mg2+ (5.5 mM) and incubated for 30 min at 22°C prior to addition of one-half mixture of ATPase activity or frozen at –20°C for subsequent SDS-polyacrylamide gel electrophoresis (PAGE). DTNB cross-linking was carried out by incubating ECF1 or ECF1F0 at 1 mg/ml in Buffer A with 200 μM DTNB for 1 h at 22°C. The DTNB-treated samples were divided into two aliquots, and 20 mM DTT was added to one, before incubating both for 30 min at 22°C. Samples were then assayed for ATPase activity and ACMA fluorescence quenching and subjected to SDS-PAGE.

ECF1 or ECF1F0 was reconstituted with 200 μM BM following the same procedures used for DTNB. The reaction was stopped after 1 h by a 30-min incubation with 20 mM DTT at room temperature.

ACMA Fluorescence Quenching—Assays of ACMA fluorescence quenching were performed essentially as described in Aggerl et al. (19). ECF1 or ECF1F0 was reconstituted into egg lecithin vesicles as described before (12), except that 0.75% sodium deoxycholate was used in the column buffer, and 2 mg/ml of egg lecithin was used in the reconstitution step. These vesicles were collected by centrifugation and resuspended to a protein concentration of 20 μg/ml in 10 mM Hepes, pH 7.5, 100 mM KCl and 2 mM MgCl2, and then valinomycin (3.6 μM), ACMA (1 μM), ATP (1 mM), and nigericin (3.6 μM) were added sequentially. ACMA fluorescence was measured at 480 nm with an excitation wavelength of 410 nm in an SLM8000 fluorometer.

SDS-PAGE—Samples (5 μg) were supplemented with 20 μM NEM and incubated for 30 min at 22°C prior to addition of one-half volume of dissociation buffer (10% SDS, 100 mM Tris, pH 6.8, 30% glycerol, and 0.03% bromphenol blue) with or without reducing agent as indicated. Subunits were separated by electrophoresis on 10–22% polyacrylamide gradient gels (24). Protein bands on SDS-PAGE were visualized by staining with Coomassie Brilliant Blue R according to Downing et al. (25). Proteins were transferred from the gel to polyvinylidene difluoride (Millipore Corp.) as described previously (12). The molecular monolayer on the gel, anti-γγγγ antibody, was characterized previously (26). The rabbit antiserum to subunit ε was described by Girvin et al. (27). The mouse monoclonal antibody to γ anti-γγγγ, was characterized previously (26).

RESULTS

Characterization of ECF1 and ECF1F0 Isolated from the Mutant γY205C—ECF1 isolated from the mutant γY205C contained a, β, γ, δ, and ε subunits in the same relative amounts as wild-type strain, whereas ECF1F0 isolated from the mutant had the same subunit composition as wild type based on SDS-polyacrylamide gel electrophoresis (see later).

The ATPase activity of ECF1 isolated from the mutant γY205C was around 3.5-fold higher than for wild-type enzyme (Table I). This is due to an altered binding of the inhibitory ε subunit, as demonstrated by the concentration dependence of the inhibition of ECF1F0 (16) by purified ε subunit. ECF1F0 from

1 The abbreviations used are: ECF1, soluble portion of the E. coli F, F0 ATP synthase; ECF1F0, membrane-bound portion of the E. coli F, F0 ATP synthase; ECF1F0, E. coli F, F0 ATP synthase; ACMA, 9-aminol-6-chloro-2-methoxy-4-nitroacetohydroxamic acid; DTNB, 5,5-dithiobis-(2-nitrobenzoic acid); DTT, dithiothreitol; MOPS, 3(N-morpholino)propanesulfonic acid; AMP-PNP, 5'-adenylyl-β,γ-imidodiphosphate; PAGE, polyacrylamide gel electrophoresis; NEM, N-ethylmaleimide; mAb, monoclonal antibody; BM, benzophenone maleimide; TF-PAM-3 tetrafluorophenylazide maleimide-3.
the mutant γY205C showed half-maximal inhibition at a concentration of 16 nM of added pure ε subunit, compared with only 9 nM for ECF1 Y205C from wild type (result not shown). ATPase activity was also measured in ECF1F0 isolated from the mutant γY205C. In the intact ATP synthase, the rate of ATP hydrolysis was essentially the same as that of wild-type enzyme (Table I).

ECF1F0 isolated from the mutant γY205C showed ATP hydrolysis linked to proton translocation, but this was somewhat less efficient than observed for wild-type enzyme under equivalent conditions (see below), implying that the mutation γY205C caused some disruption of the F1-F0 interface. ECF1F0 from the mutant also showed a significantly lower sensitivity to dicyclohexylcarbodiimide than wild-type enzyme (Table I).

**Table I. ATPase activity of ECF1 or ECF1F0**

|        | ECF1 | ECF1F0 |
|--------|------|--------|
| Activity | 36 (5) | 68 |
| Activity | 65 | 69 (6) |
| Activity | 22 | 25 |
| Wild-type | 73 (2) | 73 (2) |

**Reaction of γCys-205 with Maleimides under Different Nucleotide Conditions**—The Cys introduced at residue 205 of the γ subunit was reactive to a variety of maleimides in both ECF1 and ECF1F0, isolated from the mutant γY205C. As shown in Table I, reaction of γCys-205 in ECF1 with CM or TFPAM-3 (without photolysis) resulted in an increase in activity such that with these bulky maleimides, the modified enzyme had the same activity as ε-free ECF1 (from either the mutant γY205C or wild-type enzyme). After reaction of ECF1 Y205C from the mutant γY205C with CM to modify the introduced Cys, there was essentially no inhibition of ATPase activity on addition of a large excess of purified ε subunit. The Cys at residue 205 was also reactive to maleimides in ECF1F0, but in this case, the modification did not activate the enzyme significantly (Table I).

The rates of incorporation of [14C]NEM and the fluorescent maleimide CM into γCys-205 were compared under different nucleotide conditions. In ECF1 and ECF1F0, the rate of modification was as fast as in AMP-PNP or wild-type enzyme. After reaction of ECF1 Y205C with CM for 2 min under two nucleotide conditions (Mg2+/ADP/Pi), there was no nucleotide dependence in the ATPase activity of ECF1 F0 (result not shown). At the times (min) indicated, addition of 10 μM DTT was made to quench the unreacted CM. **Fig. 1A** shows the rate of incorporation of CM into γCys-205 of ECF1, ECF1F0, and ECF1 Y205C (result not shown). There were additional cross-linked products in small amounts compared with ADP + P1 + Mg2+ (right side) (result not shown). Fig. 1B shows the rate of modification of γCys-205 in ECF1, ECF1F0, and ECF1 Y205C. In ECF1 Y205C, there was no nucleotide dependence in the rate of modification of γ Cys-205. With ADP + P1 + Mg2+ bound, the rate of modification was as fast as in AMP-PNP + Mg2+.

Studies with Reconstituted ECF1F0 Establish the Proximity of γ Cys-205 to the Polar Loop Region of the c Subunits—F0, purified from the γ subunit mutant γY205C was reconstituted with membrane vesicles containing F0 ε subunit from the γ subunit mutants εA39C, εQ42C, εP43C, and εD44C (see "Experimental Procedures"). Cross-linking within the reconstituted ECF1F0 complex was induced by the addition of Cu2+ (1,10-phenanthroline). Fig. 2 shows the results obtained using a hybrid enzyme ECF1(γY205C)F0(cQ42C). The oxidizing agent generated a cross-link between the γ subunit and ε subunit of apparent Mr 38,000 that was readily observed in Coomassie Blue-stained gels. Based on the disappearance of the γ subunit, the yield of cross-linked product was 95%. No cross-linked product involving γ and ε was observed in ECF1F0 from the single mutant γY205C or in ECF1F0 from the single mutant εQ42C after Cu2+ treatment. The Mr 38,000 product was lost and the γ subunit (monomer) reappeared in the gel profile when Cu2+ treated samples were incubated with DTT prior to electrophoresis (result not shown), confirming that the cross-link is a disulfide bond between the introduced Cys in γ and that in the ε subunit.

As shown by the Western blot in **Fig. 2B**, there was also Cu2+ induced generation of the Mr 38,000 cross-linked product of γ and ε subunits in the hybrid mutants ECF1(γY205C)F0(cP43C) and ECF1(γY205C)F0(cD44C). This product was lost on the addition of DTT with both mutants. No cross-linking between the γ and ε subunits was obtained using the hybrid enzyme ECF1(γY205C)F0(cA39C) (result not shown). There were additional cross-linked products in small amounts con-
taining γ and c, particularly in the mutant ECF₁(γY205C)F₀(cD44C) (see Fig. 2B), some of which did not disappear with DTT treatment. These probably involve the Cys on the c subunit with remaining Tyr residues in the γ subunit region 205–228, particularly Tyr-207. One band migrating just above the main γ-c product could be a cross-link between γ and two c subunits, one via a Cys-Cys linkage involving Cys-205, the second a Cys-Tyr linkage to Tyr-207.

The Western blotting data in Fig. 2B show that with all three hybrid mutants, Cu²⁺ also induced considerable subunit c dimer formation. Dimers of subunit c have been observed in the studies of Zhang and Fillingame (18), as well as in our previous studies using mutants with Cys residues in the polar loop region of the c subunit (12).

ATPase activities were measured before and after cross-linking. For the mutant ECF₁(γY205C)F₀(cQ42C), a 95% cross-linking of γ to c caused at most a 30% inhibition of ATP hydrolysis. This inhibition is most likely partly due to subunit c dimer formation, because there was a similar loss of ATPase activity when ECF₁F₀ from the single mutant cQ42C was reacted with Cu²⁺ (result not shown).

Cross-linking of the γ subunit to a c subunit could also be induced by adding DTNB instead of Cu²⁺. Fig. 3 shows data obtained with the double mutant γY205C:cQ42C. In the experiment in Fig. 3A, the yield of γ-c cross-linked product was 50% based on the disappearance of γ (lane 2), compared with a 95% cross-linking yield in the double mutant when Cu²⁺ was used (lane 3). Note that a small amount of cross-linking of γ to c occurs in the absence of DTNB, probably due to oxidation reactions during sample preparation and incubations. Western blotting results in Fig. 3B confirm the cross-linking and show the greater specificity of DTNB in generating the γ-c cross-linked product over the subunit c dimer even under conditions where the γ-c product obtained with Cu²⁺ is in nearly the same yield as with DTNB. We assume that at the levels of DTNB used, Cys residues in the c subunit are modified more rapidly than the Cys at position 205 in the γ subunit. With most or all of the Cys of c modified, disulfide bond formation between these sites is prevented. One of the DTNB modified c subunit monomers then reacts with unmodified γ Cys-205 to generate the γ-c cross-linked product.

Table II lists the ATPase activities of the mutant after reaction with DTNB. Reaction of the double mutant with DTNB to generate around a 50% yield of cross-linked product γ-c caused an approximately 10–15% reduction in ATP hydrolysis. This compares with an approximately 40% inhibition of ATPase activity for ECF₁F₀ from the single mutant cQ42C but no inhibition of ECF₁F₀ from the mutant γY205C after the identical DTNB treatment. Apparently, in the double mutant, the modification of both γ Cys-205 and c Cys-42 in some enzyme molecules complexes for the inhibition due to DTNB reaction with c Cys-42 alone.

A Cys At Position 205 of the γ Subunit Forms Disulfide Bonds with Cys Residues Introduced at Positions 38 or 43 of the e Subunit—In the course of our recent studies, we have made several mutants containing a Cys in the e subunit (14, 16). These include εS10C, εT38C, εT43C, εS65C, and εS106C. ECF₁⁺ (the α₁β₃γ complex) isolated from the mutant γY205C was combined with each of the above-listed ε subunit mutants to examine the proximity of Tyr-205 to the ε subunit. In each case, the mutant ε subunit was added in a 10-fold molar excess to ensure full occupancy of the ε binding site. Samples were treated with 200 μM Cu²⁺ to induce disulfide bond formation. This led to cross-linking of ε to the γ subunit in the hybrids made with εH38C and εT43C but not with εS10C, εS65C, or εS108C (result not shown). This cross-linking of γ to ε had little effect on the rates of ATP hydrolysis.

Cross-linking studies were also conducted in ECF₁F₀. For these experiments, ECF₁F₀ from wild type was stripped of F₁ and these F₀-containing membranes were then reconstituted with the α₁β₃γ complex from the mutant γY205C, along with purified δ subunit and one of the different ε subunit mutants. After the reconstitution step, membranes were separated from excess δ and ε subunits prior to addition of Cu²⁺. There was Cu²⁺-induced cross-linking of the γ to the ε subunit in hybrids containing the mutations εH38C (maximum yields 50%) and εT43C (yields as high as 90%) but not with the other ε subunit mutants. As in ECF₁⁺, the cross-linked product γ-ε ran very close to the β subunit in SDS-PAGE and was best observed in Western blotting experiments, such as in Fig. 4 (A and B). The monoclonal antibody anti-γIII was used for analysis (Fig. 4A).

This mAb, like the others we have obtained to the γ subunit, reacted close to Tyr-205 (see Ref. 29), giving only a weak reaction with the γ-c cross-linked product. As a result, high concentrations of the mAb had to be used and mAb γIII then cross-reacted with the α subunit. Nevertheless, Fig. 4 (A and B) shows clearly the disappearance of γ and ε and appearance of the γ-ε cross-linked product in ECF₁F₀ containing γ Cys-205 and ε Cys-38 (lanes 3) or ε Cys-43 (lanes 4). The yield of cross-linking was estimated from Coomassie Brilliant Blue-stained gels.
4C shows data for Cu\(^{2+}\)-induced cross-linking of ECF\(_{1}\)\(_{F_{0}}\) containing the γY205C and εT43C mutations. The yield of cross-linking in this experiment, calculated from the change in area of the γ subunit band using the ε subunit band as a control, was 55%. This was accompanied by a 20% loss of ATPase activity.

Effect of Chemical Modification of γ Cys-205 and Cross-linking of γ to ε or c Subunits on Proton Pumping—ECF\(_{1}\)\(_{F_{0}}\) from the γY205C mutant or the mutant cQ42C showed a reduced proton pumping activity compared with wild-type enzyme, when measured by the ACMA fluorescence quenching assay (compare Fig. 5, traces A and B). Modification of the introduced Cys-205 with several maleimides (result not shown) or by DTNB reduced the levels of proton pumping by the mutant further. With DTNB reaction, proton pumping activity was partly recovered on adding DTT (Fig. 5A). Maleimide or DTNB modification of ECF\(_{1}\)\(_{F_{0}}\) isolated from the mutant cQ42C also had a small effect on the proton pumping activity (Fig. 5B), whereas DTNB modification of the double mutant γY205C:cQ42C caused essentially full loss of ATP-driven proton pumping (Fig. 5C), which was also mostly recovered on DTT addition. This essentially full inhibition occurred with around a 50% yield of cross-linking of γ to c, suggesting that it was the chemical modification of both sites in the complex rather than the cross-linking that was responsible. As shown by Fig. 5 (trace D), reaction of the mutant γY205C:cQ42C with benzophenone maleimide likewise caused full loss of proton pumping activity. This reagent reacts with the introduced Cys residues but does not induce disulfide bond formation. The proton pumping activity of benzophenone maleimide-modified enzyme was not recovered upon the addition of DTT.

Attempts were made to measure the proton pumping activity after Cu\(^{2+}\) treatment of various double mutants including both the Cys at γ 205 and mutations in the ε or c subunits described above. The combination of mutations γY205C and εH38C was found to abolish ATP-driven proton pumping, whereas each mutation individually retained this activity. Cu\(^{2+}\) treatment to induce cross-linking greatly reduced the proton pumping activity of all mutants tested under conditions where ATPase activity was retained. However, this treatment inhibited proton pumping activity to 80% even in wild-type enzyme, an inhibition that was only partly regained by incubation with DTT. As a consequence, the effect of cross-linking on proton pumping was not pursued further.

**DISCUSSION**

The studies described here extend our original finding (12) that oxidizing conditions generate a covalent cross-link between a Cys introduced at position 44 of the c subunit of the F\(_{0}\) and the γ subunit of the F\(_{1}\) part of ECF\(_{1}\)\(_{F_{0}}\). Here Tyr-205, one of four Tyr residues in the short stretch of the γ subunit identified as involved in the cross-linking reaction with the c subunits, has been replaced by a Cys. ECF\(_{1}\) from the mutant,
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γ205C, had an increased ATPase activity due to an altered affinity of the αβγ core complex for the inhibitory ε subunit, but the ATPase activity of the intact ECF1F0 was not altered compared with wild type by the mutation. The Tyr → Cys change also caused a small reduction in efficiency of ATP-driven proton translocation when measured by the ACMA quenching assay, but the ECF1F0 clearly retained this coupling function.

A key finding of the present study is that the Cys in γ at position 205 can be cross-linked to Cys residues introduced in the ε subunits at positions 42, 43, or 44 (30)) at Cys-87 (by cross-linking and fluorescence changes of probes attached at Cys introduced at position 8 (30)) at Cys-87 (by changes in reactivity to maleimides (31)) and at position 106 (from fluorescence changes of CM bound to a Cys at this site (30)). The results for γ Cys-205 now establish that the conformational change occurring with ATP hydrolysis is propagated by the γ subunit from the catalytic sites to the interface of the F1, with F0.

As with the conformational changes detected at other parts of the γ subunit (30, 31), the nucleotide dependence of the reaction of Cys-205 was lost on removal of the ε subunit. ECF1 from which the ε subunit has been removed is a highly active ATPase, and therefore, as we have discussed before (30, 31), the conformational changes observed at residues 8, 87, 106, and now 205 cannot be necessary for ATP hydrolysis. Rather, they likely represent structural changes that are part of the energy coupling within the complex that are in addition to rotation of the γ and ε subunits that occurs during the cooperative functioning of catalytic sites (32–34).

The covalent linkage of γ via Cys-205 to the ε subunits at positions 42, 43, or 44 proved to have very little effect on ATPase activity. A pattern of effects of covalent cross-linking of subunits to one another on enzyme function is emerging. Cross-linking of γ or ε to α or β subunits in our studies has in all cases fully inhibited ATPase activity (14–16, 35) (also see Ref. 36). In contrast, cross-linking of γ to ε (14, 16) and now of γ to ε subunits did not greatly reduce ATP hydrolysis. The implication is that the rotations or translocations of subunits that are a part of the cooperativity of catalytic sites must involve the αβε domain moving relative to γ and ε plus the ε subunit oligomer. This could occur by the αβε domain rotating relative to a fixed unit of the rest of the ATP synthase or the γ, ε, and ε subunit oligomers rotating within a scaffold of the αβε domain plus the δ, α, and β subunits.

It is not clear at present whether covalent cross-linking of γ to the ε subunit oligomer alters proton pumping. It was not possible to assess this issue when Cu²⁺ oxidation was used to generate cross-links. After DTNB-induced cross-linking, there was complete loss of proton pumping without concomitant loss of ATPase activity, implying an uncoupling of the two functions. However, it was the maleimide modification of both γ Cys-205 and the adjacent Cys in the ε subunits that caused the effect, not the cross-linking reaction per se.

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