Conformational Changes in the Escherichia coli ATP Synthase (ECF$_1$F$_0$) Monitored by Nucleotide-dependent Differences in the Reactivity of Cys-87 of the γ Subunit in the Mutant βGlu-381 → Ala*

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Cys-87, one of two intrinsic cysteines of the γ subunit of the Escherichia coli ATP synthase (ECF$_1$F$_0$), is in a short segment of this subunit that binds to the bottom domain of a β subunit close to a glutamate (Glu-381). Cys-87 was unreactive to maleimidides under all conditions in wild-type ECF$_1$ and ECF$_1$F$_0$ but became reactive when Glu-381 of β was replaced by a cysteine or alanine. The reactivity of Cys-87 with maleimidides was nucleotide-dependent, occurring with ATP or ADP + EDTA in catalytic sites, in the presence of AMP-PNP + Mg$^{2+}$ but not with ADP + Mg$^{2+}$ bound, whether F$_0$ was present or not, and not when nucleotide binding sites were empty. Binding of N-ethylmaleimide had no effect, whereas 7-diethyl-amino-3-(4-maleimidophenyl)-4-methylcoumarin increased the ATPase activity of ECF$_1$F$_0$, more than 2-fold by reaction with Cys-87. In ECF$_1$F$_0$, these reagents inhibited activity. The nucleotide dependence of the reactivity of Cys-87 of the γ subunit depended on the presence of the e subunit. In e subunit-free ECF$_1$, maleimidides reacted with Cys-87 under all nucleotide conditions, including when catalytic sites were empty. These results are discussed in terms of nucleotide-dependent movements of the γ subunit during functioning of the F$_1$F$_0$-type ATPase.

F$_1$F$_0$-type ATPases catalyze oxidative or photo-phosphorylation by using a transmembrane proton motive force to drive ATP synthesis (reviewed in Senior, 1988; Futai et al., 1989; Hatefi, 1993). In the reverse direction, these enzymes use ATP hydrolysis to generate a proton gradient that can be used in ion transport processes. The simplest F$_1$F$_0$-type ATPases are found in bacteria. The hydrophobic F$_1$ part of the Escherichia coli enzyme (ECF$_1$) contains five different subunits in the stoichiometry α3β2γδε and six, whereas the membrane-integrated F$_0$ part (ECF$_0$) contains three different subunits in the molar ratio α$_1$β$_2$ε$_{10}$. As first demonstrated by electron microscopy studies (Tiedge et al., 1985; Gogol et al., 1989a, 1989b), the three α and three β subunits alternate in a hexagonal arrangement surrounding a central cavity containing the γ subunit. The recent resolution structure of the beef heart F$_1$ (MF$_1$) confirms this arrangement and shows that the part of the γ subunit within the αβε$_3$ domain is in the form of two large α helices, one provided by residues 1-45 (ECF$_1$ numbering system) and the other by residues 223-286 (Abrahams et al., 1994). A third α helix of the γ subunit has been resolved in the x-ray analysis (Abrahams et al., 1994). This short segment of residues 82-99, including an intrinsic Cys residue (Cys-87), binds to the so-called DELSEED region (residues 380-386) of one of the β subunits.

Recent evidence indicates that the γ subunit runs from within the αβε$_3$ domain of the F$_1$ through the stalk region that connects the F$_1$ to F$_0$ (Gogol et al., 1987; Lücken et al., 1990) and binds to the c subunits of the F$_0$ that are a part of the proton channel (Watts et al., 1995). It is now generally agreed that energy coupling within the F$_1$F$_0$ complex is by conformational changes involving the stalk-forming subunits, including the γ subunit (reviewed in Boyer, 1993; Capaldi et al., 1994). Previously, we have provided evidence of nucleotide-dependent conformational changes in the γ subunit around Cys residues site-directed into positions 8 and 106 of this subunit (Aggeler and Capaldi, 1992, 1993; Turina and Capaldi, 1994a, 1994b). Here, we describe studies in which one of the two intrinsic Cys residues of the γ subunit is reacted with various maleimidides in both ECF$_1$ and ECF$_1$F$_0$. This residue, shown to be Cys-87, is shielded in wild-type enzyme but becomes available for reaction in ECF$_1$ (and ECF$_1$F$_0$) when Glu-381 of the β subunit is replaced by a smaller side chain, e.g. by a Cys or an Ala. The interaction of the short α helix of γ with the DELSEED region is shown to be nucleotide-dependent and, as with ATP hydrolysis-driven structural changes already observed at residues 8 or 106, requires binding of the ε subunit.

EXPERIMENTAL PROCEDURES

Materials—CM and BM were obtained from Molecular Probes; Sephadex G-50 was purchased from Pharmacia Biotech Inc.; all other chemicals were of analytical grade and obtained from Sigma.

Plasmids and Bacteria Strains—Routine cloning was carried out in XL1-Blue and site-directed mutagenesis in CJ 236 according to Kunkel et al. (1987). Mutant ATPase and ATP synthase was isolated from AN888 (unc), transformed with unc operon containing plasmids.

The Cys residue at position 87 of the γ subunit was replaced with Ser by using M13mp18 that contained the 1.01-kb XhoI fragment (Aggeler and Capaldi, 1992) and the oligonucleotide GACCGTGGTTT-GACGGTGTTTG. Successful mutagenesis was shown by testing for the newly created BsrBI restriction site. The mutation was incorporated in an unc operon-containing plasmid in two steps. (i) The 1.1-kb Sfu/EcoRI fragment from M13mp18 was inserted in the pBluescript derivative pRA13 (Aggeler et al., 1995). (ii) The 2.8-kb SstI/XhoI fragment of this plasmid was then introduced in pRA134 (Aggeler et al., 1995), creating pRA149 with the mutation β381C/C387S/S108C. The Glu in position 381 of the β subunit was replaced with an Ala by using M13mp18 that contained the 1.01-kb NcoI insert, described in Aggeler et al. (1992), and the oligonucleotide TCTTCCAGAACT- GCATCCATACC (nucleotide A was introduced to obtain a new NsiI restriction site for analysis). The NcoI fragment was introduced in
pRA13 and then the 5.8-kb XhoI/NsiI fragment in pRA100 (Aggerel et al., 1992), creating pRA155 with the mutation βE381A.

The mutants βE381C and βE381C/S108C have been described already (Aggerel et al., 1995).

Preparation of ECF1, and ECF1/F0—ECF1 was isolated by a modification of the method of Wise et al. (1981) described in Gogol et al. (1989b). The enzyme was precipitated for 4 h at 4 °C in 70%(NH₄)₂SO₄, pelleted by centrifugation at 10,000 × g for 15 min, and the protein then dissolved in 50 mM MOPS, pH 7.0, 0.5 mM EDTA, and 10% glycerol (v/v). Loosely bound nucleotides were removed by passing samples of enzyme through two consecutive centrifuge columns (Sephadex G-50, fine, 0.5 × 5.5 cm) (Penefsky, 1977) equilibrated in the same buffer. The resulting ECF1 preparations retain 1.6–1.8 mol of ADP + ATP bound in noncatalytic sites (see also Haughton and Capaldi, 1995). ECF1/F0 was prepared according to Foster and Fillingame (1979) with modifications described in Aggerel et al. (1987). This ATP synthase was reconstituted into egg-lecithin vesicles by the method described in Aggerel et al. (1995).

Maleimide Reaction of ECF1, and ECF1/F0—For modification by maleimides, nucleotide-depleted ECF1 (2–3 μM) was equilibrated at room temperature in 50 mM MOPS, pH 7.0, 0.5 mM EDTA, and 10% glycerol (v/v) buffer for 0.5–1 h. After addition of nucleotide, as stated, the enzyme was incubated for 5 min before the various maleimides were added. Samples were incubated in the dark at room temperature and at specific time intervals, aliquots withdrawn, and the reaction quenched by the addition of 20 mM DTT. The reaction of ECF1, with maleimides was done similarly, except in 50 mM MOPS, pH 7.0, 0.5 mM EDTA, 10% glycerol, in the presence of different nucleotides as indicated, using 20 μM of the maleimide. Data were analyzed as described in Haughton and Capaldi (1995).

Other Methods—ATPase activity was measured with a regenerating system described by Lötcher et al. (1984). α-Depleted ECF1 was prepared according to Dunn (1986) but using Sephacryl S300 (Pharmacia) followed by two passages through an α-monoclonal antibody affinity column. Protein concentrations were determined with the BCA protein assay (Pierce Chemical Co.). SDS-polyacrylamide gel electrophoresis was performed with a 10–18% SDS-containing gradient gel (Laemmli, 1970). Protein bands on gels were stained with Coomassie Brilliant Blue R (Downer et al., 1976).

RESULTS

Cys-87 of the γ subunit shows a Nucleotide-dependent Reactivity with Maleimides When Glu-381 of β Is Mutated to a Smaller Residue—In earlier studies, using the mutant βE381C, we had noted that an intrinsic Cys of the γ subunit under different nucleotide conditions. As shown in Fig. 1, there was incorporation of [³⁴ClNEM into the γ subunit of ECF1 isolated from the mutant βE381C when the reaction was carried out in EDTA alone. In the presence of nucleotide, Cys-87 of the γ subunit was not incorporated into the γ subunit in ECF1 isolated from the mutant βE381C/S108C, but the incorporation of NEM was higher in ECF1/F0 from the mutant βE381C/F0 than in ECF1 from wild-type. These results suggest that the incorporation of NEM into the γ subunit is dependent on the nucleotide conditions, and that the incorporation of NEM into the γ subunit is higher in ECF1/F0 than in ECF1 from wild-type.

To explore the reactivity of Cys-87 more fully, the mutant βE381A was prepared. This change preserves the short side chain but avoids a maleimide-reactive cysteine in the β subunit. The reactivity of Cys-87 with CM in different nucleotide conditions is shown in Fig. 3 (lanes 1–8). There was rapid and strong incorporation of CM in EDTA + ATP, EDTA + ADP, or AMP-PNP + Mg²⁺, a low incorporation of reagent in EDTA or Mg²⁺ alone, but essentially no modification of γ with Mg²⁺ + ADP-bound, either when added directly, or as generated on the protein by addition of ATP + Mg²⁺ followed by CM labeled ECF1/F0 with maleimides. In these experiments, NEM was incorporated rapidly into the Cys at position 381 of β, as well as into the δ subunit (not shown). The δ subunit is reactive to maleimides in wild-type ECF1, but its modification (at Cys-140) has no effect on activity (Mendel-Hartvig and Capaldi, 1991; Ziegler et al., 1994). Activity measurements showed that NEM incorporation, into either the γ subunit, or into Cys-381 of β, or both, activated the enzyme more than 2-fold. In contrast, CM modification of one or both sites caused essentially full inhibition (Fig. 2B).

Recently, Duncan et al. (1995a, 1995b) used a mutant βD380C/Y87S to distinguish which of the two Cys in the γ subunit (Cys-87 or Cys-112) was involved in disulfide bond cross-linking between γ and the β DELSEED region. Following the same approach, we constructed the mutant βE381C/Y87S/ S108C to identify which of the intrinsic Cys in γ was being reacted by maleimides. There was reactivity of the β subunit in the Cys at 381 and modification of both δ and ε (via Cys-108) but no labeling of the γ subunit by CM in this mutant (Fig. 3, lanes 10 and 11). Therefore, Cys-87 must be the site of maleimide incorporation into the γ subunit. CM modification of the mutant βE381C/Y87S/S108C reduced the ATPase activity by 90% (Fig. 2C). This inhibition is not due to modification of Cys-140 as discussed above. Moreover, CM modification of the ECF1 isolated from mutant S108C had no effect on activity (result not shown). Therefore, it must be modification of Cys-87 in the DELSEED region of the β subunit that caused the observed inhibition of activity in this mutant.
enzyme turnover. Reaction of the γ subunit with NEM in enzyme from the mutant βE381A had been freed of ε subunit by affinity chromatography with a monoclonal antibody against the ε subunit (Dunn, 1986). Fig. 4 shows that Cys-87 is labeled by CM under all nucleotide conditions including ADP + Mg2+ or AMP-PNP + Mg2+. This site was also labeled by CM in EDTA or Mg2+ alone (results not shown). The ATPase activity of the ε-free ECF1 from mutant βE381A was high, i.e. 70 μmol of ATP hydrolyzed per min per mg. There was no significant increase in the activity on reaction of CM (Fig. 2E), in contrast to the activation observed with enzyme that had not been freed of ε subunit.

In another set of experiments, the effect of removing the ε subunit on the inhibition of ATPase activity by CM was investigated in the mutant βE381CγC875SγS108C. As shown in Fig. 2F, CM inhibited ε-free ECF1 from this mutant. Therefore, it is the interaction between β and γ, rather than between β and ε, which is perturbed when the Cys at residue 381 of β is reacted with CM.

Cys-87 is Reactive to Various Maleimides in ECF1. ECF1 from the Mutant βE381A—ECF1F0 purified from the mutant βE381A had normal ATPase activity, i.e. around 20 μmol of ATP hydrolyzed per min per mg protein, which was inhibited to 90% by 50 μM dicyclohexylcarbodiimide, results similar to those obtained with wild-type enzyme. Reaction of this preparation with NEM, CM, or BM all led to an inhibition of activity, with NEM in the dark at room temperature for 6 h in 50 mM MOPS, pH 7.0, 0.5 mM EDTA, 10% glycerol, after addition of 2.5 mM ATP (lane 1), 2 mM Mg2+ + 2.5 mM ATP (lane 2), 2 mM Mg2+ + 2.5 mM ATP (lane 3), without additions (lane 4), 2 mM ADP (lane 5), 2.5 mM ATP (lane 6), 2 mM AMP-PNP (lane 7), 2 mM Mg2+ + 2 mM AMP-PNP (lane 8). ECF1 from the mutant βE381CγC875SγS108C treated as for βE381A was reacted with 100 μM CM for 6 h in the presence of 2.5 mM ATP (lane 10) or 2 mM Mg2+ + 2.5 mM ATP (lane 11). The reaction was quenched by 20 mM DTT. Lanes 1–8, 10, and 11 contain 50 μg of protein. Lane 9 contained molecular weight markers. The left side shows Coomasie Blue staining and the right side shows the fluorescence pattern.

Effect is due to reaction of Cys-87 and not Cys-140 of the δ subunit. The highest amount of inhibition was with the BM (more than 90%).

**Discussion**

Cys-87 is at the end of a short α helix of the γ subunit that interacts with the so-called DELSEED region of the β subunit (Abrahams et al., 1994). We have found that Cys-87 can be cross-linked in essentially 100% yield by disulfide bond formation to a Cys replacing Glu at 381 (Aggeler et al., 1995), indicating the close proximity of the two residues, consistent with the ~4 Å spacing from side chain S to S, estimated from the x-ray structural data (Abrahams et al., 1994). Cys-87 is buried in wild-type ECF1 and ECF1F0. However, when Glu-381 of the β subunit is exchanged for a smaller and uncharged side chain, such as Cys or Ala, this residue of γ becomes exposed for reaction with maleimides at least as large as CM. This exposure is nucleotide-dependent.

In enzyme from which catalytic site nucleotide has been removed, Cys-87 is essentially buried. Addition of nucleotide, either ADP or ATP in the presence of EDTA, exposes Cys-87 for reaction with various maleimides. In the absence of Mg2+, the binding constants for nucleotide in each of the three catalytic sites, including that in the β which is linked to the short α helix of γ, is around 100 μM (Weber et al., 1994; Grüber and Capaldi, 1996), similar to that of isolated β subunit, suggesting an open arrangement of the sites (as in β3 in the structure of MF5). In the presence of Mg2+, Cys-87 is exposed when ATP is bound, as demonstrated by the data for AMP-PNP, but the residue is buried in ADP or ADP + P1. It appears, therefore, that the short α helix undergoes a release or reorganization that exposes Cys-87 when the catalytic sites are all open, or when ATP is bound, and that this is reversed on ATP hydrolysis.

A conformational change of the γ subunit related to ATP binding and hydrolysis has been seen previously by changes in cross-linking from a Cys introduced at position 8 of the γ subunit (in the long N-terminal α helix) with the β subunit(s) (Aggeler and Capaldi, 1993). This process has also been followed by fluorescence changes of CM bound to either the Cys introduced at position 8 or another Cys introduced at residue 106 of the γ subunit (Turina and Capaldi, 1994a, 1994b). Fluorescence measurements under anisotropic catalysis conditions showed that the conformational change in the γ subunit occurs with bond cleavage of ATP to product ADP-Pi, rather than with P1 release (Turina and Capaldi, 1994a, 1994b).

Importantly, the conformational rearrangements observed here by changes in the reaction of Cys-87 were lost on removal of the ε subunit. Without the ε subunit bound, Cys-87 is ex-
posed for reaction under all nucleotide conditions, including when catalytic sites are empty. The conformational changes observed for Cys-8 by cross-linking and from both Cys-8 and Cys-106 by fluorescence measurements were also lost when the ε subunit was removed (Aggeler and Capaldi, 1993; Turina and Capaldi, 1994a). The enzyme continues to show highly cooperative ATPase activity in the absence of the ε subunit. The implication, therefore, is that the ε subunit in some way controls or regulates structural changes in the γ subunit, and these changes are likely a part of the energy transduction mechanism.

The second interesting aspect of the reactivity of Cys-87 is the effect on activity of the enzyme. When Glu-381 is replaced by an Ala, reaction of Cys-87 with NEM has very little effect in isolated ECF1 ε, whereas incorporation of CM activates the enzyme around 2.5-fold. This activation is related to ε subunit binding, as it is lost when the ε subunit is removed. By contrast, the reaction of Cys-87 in ECF1 F0 with either NEM or CM leads to inhibition of ATPase activity by 50% or more, while modification by BM induces almost full inhibition. These results for ECF1 ε and ECF1 F0 can be compared with data for CF1 and CF1 ε. In the chloroplast enzyme, the equivalent residue of Cys-89, numbered Cys-87, is reactive to NEM even with a Glu subunit present and gives the same inhibition as unmodified enzyme in "ECF1 ε-free ECF1. Rather, the effect of modification of Cys-87 seems to be a steric effect, based on the results with ECF1 F0 from the mutant βE381A, where inhibition occurs with any of the maleimides used. Studies with the mutant βE381C/C87S/S108C also point to the importance of sterical constraints for conformational changes involving the short α helix of γ and DELSEED region of β. Modification of a Cys-at 381 in the β subunit with NEM activated, whereas reaction of this site with CM causes a dramatic inhibition of activity.

Taken together, the nucleotide dependence and activity effects suggest that there is a loosening and possibly a release of the γ subunit at its catch region with a β subunit on ATP binding, which is reversed on ADP formation. Such a release, followed by rebinding, may be a part of coupling catalytic sites with the proton channel and would be a necessary step if the γ subunit moves relative to the αβγ domain, as suggested by the structural features of the enzyme (Abrahams et al., 1994) and as visualized by electron microscopy (Gogol et al., 1990) and, more recently, by biochemical methods (Duncan et al., 1995a, 1995b).

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