**Conformational Changes in the Escherichia coli ATP Synthase (ECF₁F₀) Monitored by Nucleotide-dependent Differences in the Reactivity of Cys-87 of the γ Subunit in the Mutant βGlu-381 → Ala**

(Zhaoyang Feng, Robert Aggeler, Margaret A. Haughton, and Roderick A. Capaldi)

From the Institute of Molecular Biology, University of Oregon, Eugene, Oregon 97404-1229

Cys-87, one of two intrinsic cysteines of the γ subunit of the Escherichia coli ATP synthase (ECF₁F₀), 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 maleimides under all conditions in wild-type ECF₁ and ECF₁F₀ but became reactive when Glu-381 of β was replaced by a cysteine or alanine. The reactivity of Cys-87 with maleimides was nucleotide-dependent, occurring with ATP or ADP + EDTA in catalytic sites, in the presence of AMP-PNP + Mg²⁺ but not with ADP + Mg²⁺ bound, whether F₁ was present or not, and not when nucleotide binding sites were empty. Binding of N-ethylmaleimide had no effect, whereas 7-diethylamino-3-(4-maleimidylphenyl)-4-methylcumarin increased the ATPase activity of ECF₁ more than 2-fold by reaction with Cys-87. In ECF₁F₀, these reagents inhibited activity. The nucleotide dependence of the reaction of Cys-87 of the γ subunit depends on the presence of the ε subunit. Inε-subunit-free ECF₁, maleimides 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 F₁F₀-type ATPase.

The F₁F₀-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; Hatfield, 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 F₁F₀-type ATPases are found in bacteria. The hydrophilic F₁ part of the Escherichia coli enzyme (ECF₁)1 contains five different subunits in the stoichiometry α₃β₃γδε, whereas the membrane-integrated F₀ part (ECF₀) contains three different subunits in the molar ratio a₁b₂c₁₀–₁₂.

As first demonstrated by electron microscopy studies (Tiedge et al., 1985; Gogol et al., 1989a, 1989b), the three α and three β subunits assemble in a hexagonal arrangement surrounding a central cavity containing the γ subunit. The recent high resolution structure of the beef heart F₁ (MF₁) confirms this arrangement and shows that the part of the γ subunit within the αβγ domain is in the form of two large α helices, one provided by residues 1-45 (ECF₁ 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 the β subunits.

Recent evidence indicates that the γ subunit runs from within the αβγ domain of the F₁ through the stalk region that connects the F₁ to F₀ (Gogol et al., 1987; Lücken et al., 1990) and binds to the c subunits of the F₀ that are a part of the proton channel (Watts et al., 1995). It is now generally agreed that energy coupling within the F₁F₀ 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 maleimides in both ECF₁ and ECF₁F₀. This residue, shown to be Cys-87, is shielded in wild-type enzyme but becomes available for reaction in ECF₁ (and ECF₁F₀) when Glu-381 of the β subunit is replaced by a smaller amino acid, 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 1.4-kb EcoRI/SstI fragment of E. coli (Aggeler and Capaldi, 1992) and the diglutamidodecyl GACGCTGTTT-GAGCCGCTGTTT. Successful mutagenesis was shown by testing for the newly created BsrBI restriction site. The mutation was incorporated in an uncle operon-containing plasmid in two steps. (i) The 2.8-kb SstI/XhoI fragment of this plasmid was then introduced in pRA134 (Aggeler et al., 1995), creating pRA149 with the mutation βE381C/S87C/S108C.

The Glu in position 381 of the β subunit was replaced with an Ala by using M13mp18 that contained the 1.01-kb NsiI insert, described in Aggeler et al. (1992), and the diglutamidodecyl TCTTCTCAATAC-GCTTCTAC (nucleotide A was introduced to obtain a new NsiI restriction site for analysis). The NsiI fragment was introduced in

*This work was supported by National Institutes of Health Grant HL24526 (to R. A. C.). The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked “advertisement” in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

†To whom correspondence should be addressed. Tel.: 541-346-5881; Fax: 541-346-4854.

1The abbreviations used are: ECF₁, soluble portion of the E. coli F₁F₀-ATP synthase; ECF₁F₀, E. coli F₁F₀-ATP synthase; MOPS 3-(N-morpholino)propanesulfonic acid; NEM, N-ethylmaleimide; AMP-PNP, 5’-adenyl-β,γ-imidodiphosphate; DTT, dithiothreitol; CM, 7-diethylamino-3-(4-maleimidylphenyl)-4-methylcumarin; BM, benzophenone-4-maleimide; kb, kilobase pair(s).

1 The abbreviations used are: ECF₁, soluble portion of the E. coli F₁F₀-ATP synthase; ECF₁F₀, E. coli F₁F₀-ATP synthase; MOPS 3-(N-morpholino)propanesulfonic acid; NEM, N-ethylmaleimide; AMP-PNP, 5’-adenyl-β,γ-imidodiphosphate; DTT, dithiothreitol; CM, 7-diethylamino-3-(4-maleimidylphenyl)-4-methylcumarin; BM, benzophenone-4-maleimide; kb, kilobase pair(s).
pRA13 and then the 5.8-kb XhoI/NsiI fragment in pRA100 (Aggeler et al., 1992), creating pRA155 with the mutation βE381A.

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

Preparation of ECF1 and ECF1F0—ECF1 was isolated by a modification of the method of Wise et al. (1981) described in Gogol et al. (1988a). The enzyme was precipitated for 1 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). ECF1F0 was prepared according to Foster and Fillingame (1979) with modifications described in Aggeler et al. (1987). This ATP synthase was reconstituted into egg-lecithin vesicles by the method described in Aggeler et al. (1995).

Maleimide Reaction of ECF1 and ECF1F0—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, 5 mM MgCl2, and 10% glycerol. Labeling of ECF1 from the mutant βE381C with [14C]NEM (DuPont NEN) was conducted 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 Lötsher et al. (1984). β-Depleted ECF1 was prepared according to Dunn (1986) but using Sephacyr S300 (Pharmacia) followed by two passages through an ε-monomoclonal 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 was reactive to various maleimides. This preliminary observation was followed up as an approach to examining the conformation of the γ subunit under different nucleotide conditions. As shown in Fig. 1, there was incorporation of [14C]NEM into the γ subunit of ECF1 isolated from the mutant βE381C when the reaction was carried out in EDTA alone (no nucleotide in catalytic sites) or in ADP + Mg2+ + Pi. 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-108) has no effect on activity (Mendel 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 βE380C/C875S 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/C875S/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/C875S/S108C reduced the ATPase activity by 90% (Fig. 2C). This inhibition is not due to modification of Cys-3140 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-381 in the DELSEED region of the β subunit that caused the observed inhibition of activity in this mutant.

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 under 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...
enzyme turnover. Reaction of the γ subunit with NEM in enzyme from the mutant βE381A had no effect on activity. In contrast, CM modification caused almost 2.5-fold activation of the ATPase activity (Fig. 2D) compared with almost full inhibition when both Cys-87 and the Cys at residue 381 of β were modified.

Nucleotide Dependence of the Reactivity of γ Cys-87 Is Lost in c-Free ECF₁—The reactivity of CM was monitored in ECF₁ from the mutant βE381A that had been freed of γ subunit by affinity chromatography with a monoclonal antibody against the γ subunit (Dunn, 1986). Fig. 4 shows that Cys-87 is labeled by CM under all nucleotide conditions including ADP + Mg<sup>2+</sup> or AMP-PNP + Mg<sup>2+</sup>. This site was also labeled by CM in EDTA or Mg<sup>2+</sup> 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 βE381C/γC875S/S108C. 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 from the Mutant βE381A—ECF₁-Fₐ 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 CM gave a similar pattern of labeling to that with ECF₁ from this mutant, i.e. strong labeling of the γ subunit in AMP-PNP + Mg<sup>2+</sup>, but little or none in Mg<sup>2+</sup> alone, or in ADP + Mg<sup>2+</sup> (result not shown). There was also some reaction of the reagent with the δ subunit, but no significant reaction of the intrinsic Cys in the b subunit under the labeling conditions employed. Fig. 2G summarizes the effects on the ATPase activity of modification by three different maleimides. Modification by NEM, CM, and BM all led to an inhibition of activity that was not seen with wild-type ECF₁-Fₐ, indicating that the 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 ECF₁-Fₐ. 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 Mg<sup>2+</sup>, 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; Gruber and Capaldi, 1996), similar to that of isolated β subunit, suggesting an open arrangement of the sites (as in β₉ in the structure of MF₁). In the presence of Mg<sup>2+</sup>, 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 + P<sub>i</sub>. 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 unisite catalysis conditions showed that the conformational change in the γ subunit occurs with bond cleavage of ATP to product ADP-P<sub>i</sub>, rather than with P<sub>i</sub> 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, 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 ECF1F0 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 and ECF1F0 can be compared with data for CF1 and CF1F0. In the chloroplast enzyme, the equivalent residue of Cys-87, numbered Cys-89, is reactive to NEM even with a Glu substitution. However, Cys-89 occurs in thylakoid membranes when these are energized by light (i.e. when ATP is being made) and does not occur in the dark (with ADP bound) (McCarty and Fagan, 1973). This cannot explain the activity effects of ECF1, as the modification of Cys-87 by CM activates the enzyme when the ϵ subunit is present and gives the same activity as unmodified enzyme in ϵ-free ECF1. Rather, the effect of modification of Cys-87 seems to be a steric effect, based on the results with ECF1F0 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 steric constraints for conformational changes involving the short α helix of γ and DELSEED region of β. Modification of a Cys-at ϵ381 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).

Acknowledgment—The excellent technical assistance of Kathy Chicas-Cruz is gratefully acknowledged.

REFERENCES

Abrahams, J. P., Leslie, A. G. W., Lutter, R., and Walker, J. E. (1994) Nature 370, 621–625
Aggeler, R., and Capaldi, R. A. (1992) J. Biol. Chem. 267, 21355–21359
Aggeler, R., and Capaldi, R. A. (1993) J. Biol. Chem. 268, 14576–14578
Aggeler, R., and Capaldi, R. A. (1992) Biochemistry 31, 2956–2961
Aggeler, R., Haughton, M. A., and Capaldi, R. A. (1995) J. Biol. Chem. 270, 9185–9191
Boyer, P. D. (1993) Biochim. Biophys. Acta 1140, 215–250
Capaldi, R. A., Aggeler, R., Turina, P., and Wilkins, S. (1994) Trends Biochem. Sci. 19, 284–289
Downer, N. W., Robinson, N. C., and Capaldi, R. A. (1976) Biochemistry 15, 2930–2936
Duncan, T. M., Zhou, Y., Bulygin, V. V., Hutcheon, M. L., and Cross, R. L. (1995a) Trans. Biochem. Soc. 23, 736–740
Duncan, T. M., Bulygin, V. V., Hutcheon, M. L., and Cross, R. L. (1995b) Proc. Natl. Acad. Sci. U. S. A. 92, 10964–10968
Dunn, S. D. (1986) Anal. Biochem. 159, 35–42
Foster, D. L., and Fillingame, R. H. (1979) J. Biol. Chem. 254, 8230–8236
Futai, M., Noumi, T., and Maeda, M. (1989) Annu. Rev. Biochem. 58, 111–136
Gogol, E. P., Lücken, U., and Capaldi, R. A. (1987) FEBS Lett. 215, 274–278
Gogol, E. P., Aggeler, R., Sagermann, M., and Capaldi, R. A. (1989a) Biochemistry 28, 4717–4724
Gogol, E. P., Lücken, U., Bork, T., and Capaldi, R. A. (1989b) Biochemistry 28, 4709–4716
Gogol, E. P., Johnston, E., Aggeler, R., and Capaldi, R. A. (1990) Proc. Natl. Acad. Sci. U. S. A. 87, 9585–9589
Grüber, G., and Capaldi, R. A. (1996) Biochemistry 35, 3875–3879
Hafell, Y. (1993) Eur. J. Biochem. 218, 759–767
Haughton, M. A., and Capaldi, R. A. (1995) J. Biol. Chem. 270, 20568–20574
Kupke, T. A., Roberts, J. D., and Zakour, M. A. (1987) Methods Enzymol. 154, 367–382
Laemmli, U. K. (1970) Nature 227, 680–685
Löbcher, H.-R., de Jong, C., and Capaldi, R. A. (1984) Biochemistry 23, 4140–4143
Lu¨cken, U., Gogol, E. P., and Capaldi, R. A. (1990) Biochemistry 29, 5339–5343
McCarty, R. E., and Fagan, J. (1973) Biochemistry 12, 1503–1507
Mendel-Hartwig, J., and Capaldi, R. A. (1991) Biochim. Biophys. Acta 1060, 125–124
Moroney, J. V., Fullmer, C. S., and McCarty, R. E. (1984) J. Biol. Chem. 259, 7281–7285
Pfenner, S. H. (1977) J. Biol. Chem. 252, 2891–2899
Senior, A. E. (1988) Physiol. Rev. 68, 177–231
Soteropoulos, P., Ong, A. M., and McCarty, R. E. (1994) J. Biol. Chem. 269, 19810–19816
Tiedge, H., Lunsdorf, H., Schafer, G., and Schairer, H. V. (1985) Biochemistry 24, 7874–7878
Turina, P., and Capaldi, R. A. (1994a) Biochemistry 33, 14275–14280
Watts, S. D., Zhang, Y., Fillingame, R. H., and Capaldi, R. A. (1995) FEBS Lett. 368, 235–238
Weber, J., Wilke-Mounts, S., and Senior, A. E. (1994) J. Biol. Chem. 269, 20462–20467
Wise, J. G., Latchney, R. L., and Senior, A. E. (1981) J. Biol. Chem. 256, 10383–10389
Ziegler, M., Xiao, R., and Penefsky, H. S. (1994) J. Biol. Chem. 269, 4233–4239