Nucleotide-dependent Movement of the ε Subunit between α and β Subunits in the Escherichia coli F1F0-type ATPase*

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Mutants of ECF-1-ATPase were generated, containing cysteine residues in one or more of the following positions: αSer-411, βGlu-381, and εSer-108, after which disulfide bridges could be created by CuCl2 induced oxidation in high yield between α and ε, β and ε, α and γ, β and γ (endogenous Cys-87), and α and β. All of these cross-links lead to inhibition of ATP hydrolysis activity. In the two double mutants, containing a cysteine in εSer-108 along with either the DELSEED region of β (Glu-381) or the homologous region in ε (Ser-411), there was a clear nucleotide dependence of the cross-link formation with the ε subunit. In βE381C εS108C the βε cross-link was obtained preferentially when Mg2+ and ADP + Pi, (addition of MgCl2 + ATP) was present, while the αε cross-link product was strongly favored in the αS411C εS108C mutant in the Mg2+ ATP state (addition of MgCl2 + 5′-adenyl-β,γ-imidodiphosphate). In the triple mutant αS411C βE381C εS108C, the ε subunit bound to the β subunit in Mg2+ADP and to the α subunit in Mg2+-ATP, indicating a significant movement of this subunit. The γ subunit cross-linked to the β subunit in higher yield in Mg2+-ATP than in Mg2+-ADP, and when possible, i.e., in the triple mutant, always preferred the interaction with the β over the α subunit.

There has been significant recent progress in determining the structure of the F1 part of the F1F0-type ATP synthase, a key enzyme in oxidative phosphorylation and photosynthesis (Senior 1988, 1990; Boyer, 1993). The F1, which can be detached from the F0 and studied separately, is a complex of 9 subunits (nomenclature for the Escherichia coli enzyme) at the bottom of the stalk region, and alternate around a ring of 9 subunits, with a short COOH-terminal helix extending from the top NH2-terminal domain of the α and β subunits into the stalk region, and a short NH2-terminal helix running from the catalytic site domain into the stalk. These two helices form a coiled coil. A third short α-helix of the γ subunit (residues 82–99 in the E. coli enzyme) is inclined at about 45° to the two larger helices at the bottom of the F1 as it becomes the stalk. The remainder of the γ subunit is unresolved in the structure, presumably because it was disordered in the crystal form. A key feature of F1 from a functional standpoint is its asymmetry, identified earlier and clearly revealed in the x-ray structure determination. In the crystal form examined, three αβ pairs can be distinguished based upon nucleotide occupancy of catalytic sites and by interactions of the γ subunit. One β subunit (βφ), with Mg2+-AMP-PNP,1 in its catalytic site, is linked to the short α-helix of the γ subunit via the COOH-terminal domain at the sequence DELSEED (which is highly conserved in all F1-ATPases). In a second β subunit, the catalytic site is empty (βε). The β subunit and its partner α subunit (αε) have several contacts with the two long α-helices of the γ subunit. The catalytic site in the third αβ pair (αδε-βφ) contains Mg2+ADP. Overall, the interactions between the γ subunit and the ring of α and β subunits are few, and relatively nonspecific. The major contact is at the top of the molecule. Here, the COOH-terminal helix of γ is slotted into the continuous ring provided by hydrophobic loops under the β-sheet region of the six α and β subunits. Abrahams et al. (1994) have likened this region to a molecular bearing which could allow the γ subunit to rotate relative to the α and β subunits. Evidence for movements of the γ subunit relative to α-β subunit pairs has been accumulating for several years, beginning with the electron microscopy studies of Gogol et al. (1990) and has more recently included cross-linking (Aggeler and Capaldi, 1993; Aggeler et al., 1993) and fluorescence data (Turina and Capaldi, 1994). There is also clear evidence of movements of the ε subunit during the working of the enzyme (Mendel-Hartvig and Capaldi, 1991; Aggeler et al., 1992, 1995). Here, we describe experiments showing that the ε subunit can be bound at either α or β subunits, a switching that would appear to require significant rotational movements of this subunit within the αβ subunit ring.

EXPERIMENTAL PROCEDURES

Construction of Plasmids Containing Mutations in uncA, uncD, and uncC—Site-directed mutagenesis was carried out according to Kunkel et al. (1987) using Cl 236 (New England Biolabs). For routine cloning procedures (Maniatis et al., 1982; Davis et al., 1986) XL1-Blue (Strat...
The mutation in the α subunit at position 411 replacing a serine residue with cysteine was created by using the oligonucleotide TCGATTGGGATGACACCTTTA and M13mp18, that contained in the Smal site the 98-base pair SmaI fragment with the part of uncA encoding the COOH-terminal half of the α subunit, obtained from pRA100 (Aggeler et al. 1992). Successful introduction of the mutation was determined by cleavage with restriction enzyme SphI. The mutation was then inserted in a plasmid containing the unc operon: (i) the 493-base pair PmlI/SulI fragment was incorporated in pRA14 (Aggeler et al., 1995), containing the 5.8-kilobase Xhol/NsiI fragment of pRA102, which encodes the mutant ε subunit with a cysteine replacing the serine residue 308 (Aggeler et al., 1992); (ii) the 5.8-kilobase Xhol/NsiI fragment was inserted in pRA100 producing the plasmid pRA140, which contained the mutations αS108C and εS108C. A double and triple mutant were created by inserting the 2.8-kilobase Xhol/Sad fragment of pRA140 in the plasmid pRA133 or pRA134 (Aggeler et al., 1995), to obtain pRA142 or pRA143 with the mutations αS411C/εS108C and αS411C/εS108C/εS108C, respectively.

Cross-linking with CuCl2—Mutant enzymes were obtained from AN888 (unc+) containing plasmids pRA134, pRA140, pRA142, and pRA143, respectively. ECF1 and ECF1F0 were isolated as described by Gogol et al. (1989a) and Aggeler et al. (1987). Cross-linking between subunits was induced by CuCl2, as described in Aggeler et al. (1995) in buffer containing 50 mM MOPS, pH 7.0, 10 mM glycerol, 2.0–2.5 mM MgCl2, and 2 mM nucleotide. Cross-link products were analyzed by polyacrylamide gel electrophoresis (Laemmli, 1970). Gels were stained with Coomassie Brilliant Blue R (Downer et al., 1976). The identity of the subunits involved in cross-links was revealed by use of monoclonal antibodies on Western blots (Mendel-Hartvig and Capaldi, 1991). ATPase activity was measured in a regenerating system according to Gogol et al. (1984). Protein concentrations were determined with the BCA protein assay from Pierce.

RESULTS

Four different mutants were used in this study: (i) βε381C/εS108C, (ii) αS411C/εS108C, (iii) αS411C/βε381C, and (iv) αS411C/βε381C/εS108C. All grew to the same density on sucrose and showed multisite or cooperative ATPase activities similar to wild-type enzyme, i.e. 8–14 μmol of ATP hydrolyzed per min/mg.

Cross-linking Studies with the Mutant βε381C/εS108C—Previous studies had shown that addition of CuCl2 to ECF1 isolated from the mutant βε381C/εS108C catalyzed disulfide bond formation between Cys-381 of one β subunit and the intrinsic Cys-87 of the γ subunit (see “Discussion”), as well as a cross-link between Cys-381 of a second β subunit and Cys-108 of ε (Aggeler et al. 1995). Either cross-link inhibited ATPase activity essentially fully. Additional cross-links were also formed in lower yield between an as yet unidentified intrinsic Cys of α and the intrinsic Cys-140 of δ, and between Cys-381 of the third β subunit and Cys-140 of δ. It was shown that neither of the cross-links involving the δ subunit affected activity. The cross-linking between β and ε, and between β and γ subunits in the mutant βε381C/εS108C is both CuCl2 concentration dependent and sensitive to which nucleotides are present in catalytic sites, as shown in Fig. 1. With Mg2++ AMP-PNP (a non-cleavable analog of ATP) bound in the three catalytic sites (concentration 2 mM), the yield of cross-linking of β to ε is lower at all CuCl2 concentrations than with Mg2++ ADP + P, in catalytic sites. At the same time, the yield of disulfide bond formation between β and γ is higher in Mg2++ AMP-PNP than in Mg2++ ADP + P.

Cross-linking Studies with the Mutant αS411C/εS108C—The α and β subunits of F1-ATPases have a very similar fold (Abrahams et al., 1994) and residue 411 of the α subunit is the equivalent of Glu-381 in the DELSEED region of the β subunit. Treatment of ECF1 from the mutant αS411C/εS108C with CuCl2 induced disulfide bond formation between an α subunit and the ε subunit in high yield, along with a small amount of cross-linking between an α and the γ subunit (Fig. 2A). There was inhibition of ATPase activity in proportion to the yield of ε subunit Movement in E. coli F0
is seen in the mutant \( \beta E381C/\epsilon S108C \). Note that the yield of cross-linking of \( \alpha \) to \( \gamma \) is low and relatively insensitive to nucleotide conditions (although essentially full cross-linking is obtained at 200 \( \mu M \) CuCl\(_2\), Fig. 2B) indicating that this product is less favored than the \( \beta-\gamma \) cross-linked product formed in the mutant \( \beta E381C/\epsilon S108C \).

Cross-linking Studies with the Mutant \( \alpha S411C/\beta E381C/\epsilon S108C \)—The observations with mutants \( \beta E381C/\epsilon S108C \) and \( \alpha S411C/\beta E381C/\epsilon S108C \) suggest that the \( \epsilon \) subunit is able to move between two positions in a nucleotide dependent manner, one position close to \( \alpha \), the other close to a \( \beta \) subunit. This was examined further in the mutant \( \alpha S411C/\beta E381C/\epsilon S108C \). As shown in Fig. 3A, the predominant cross-linked product involving the \( \epsilon \) subunit is with the \( \beta \) subunit in Mg\(^{2+}\) ADP + P\(_i\), and with the \( \alpha \) subunit in Mg\(^{2+}\) AMP-PNP. These nucleotide dependent interactions of the \( \epsilon \) subunit were clearly observed at 5 \( \mu M \) CuCl\(_2\) within 5 min. However, in order to obtain maximal cross-linking yields, incubation times longer than 30 min were necessary. CuCl\(_2\) treatment of the mutant \( \alpha S411C/\beta E381C/\epsilon S108C \) also created a cross-link product between an \( \alpha \) and \( \beta \) subunit that was not seen in either mutant \( \alpha S411C/\epsilon S108C \) or \( \beta E381C/\epsilon S108C \), indicating that it involves disulfide bond formation between the Cys-411 of \( \alpha \) and Cys-381 of \( \beta \). This disulfide bond formation between the \( \alpha \) and \( \beta \) subunits inhibited ATPase activity in proportion to yield, based on studies with the mutant \( \alpha S411C/\beta E381C \) in which this is the exclusive cross-linked product at low CuCl\(_2\) concentrations (result not shown). At high CuCl\(_2\) concentrations, it was possible to obtain very high yields of the \( \alpha-\beta \) subunit product and, at the same time, to get essentially full cross-linking of \( \gamma \) and \( \epsilon \) in products with an \( \alpha \) or \( \beta \) subunit (Fig. 3B). The only significant triple subunit cross-link observed involved \( \alpha + \beta \) and the \( \epsilon \) subunit. Therefore, the \( \alpha-\beta \) pair that becomes internally cross-linked is different from the \( \alpha-\beta \) pair interacting with \( \epsilon \), or that pair interacting with the \( \gamma \) subunit. It may be the \( \alpha-\beta \) pair that interacts internally is the one that has the \( \delta \) subunit bound. However, it is not clear that the \( \delta \) subunit is bound at its physiological binding site in isolated \( \text{F}_1 \), which complicates the analysis.

The effect of CuCl\(_2\) treatment on \( \text{ECF}_1 \) isolated from the mutant \( \alpha S411C/\beta E381C/\epsilon S108C \) is shown in Fig. 4A. The same \( \alpha-\beta \) cross-linked product, along with cross-links involving \( \alpha \) and \( \beta \) with \( \gamma \) and \( \epsilon \), were obtained in the intact ATP synthase that were seen in isolated \( \text{ECF}_1 \). Moreover, the nucleotide dependent shifting of the \( \epsilon \) subunit between an \( \alpha \) and \( \beta \) subunit was also observed in \( \text{ECF}_1 \) (Fig. 4, B and C).

In \( \text{ECF}_1 \), there was less than full cross-linking of \( \gamma \) and \( \epsilon \) to \( \alpha \) and \( \beta \) subunits. At the same time, there appeared to be a higher yield of the \( \alpha-\beta \) cross-linked product than can be accounted for by only one \( \alpha-\beta \) pair being involved. This suggests that there is competition between cross-linking of \( \epsilon \) to \( \gamma \) to \( \alpha \) and \( \beta \) versus cross-linking between \( \alpha \) and \( \beta \) subunits, which is in favor of the latter product in the intact ATP synthase.

**DISCUSSION**

The results of CuCl\(_2\)-induced disulfide bond formation in mutants that have a Cys at position 381 of the \( \beta \) subunit and at the equivalent position, 411, in the \( \alpha \) subunit, along with a Cys in the \( \epsilon \) subunit at position 108, provide important insight into the dynamics of the structure of \( \text{ECF}_1 \) and \( \text{ECF}_1 \). They show clearly that the \( \epsilon \) subunit can move such that the COOH-terminal domain of this subunit interacts with the equivalent regions of either an \( \alpha \) or \( \beta \) subunit. The shifting of the \( \epsilon \) subunit between these subunits is nucleotide dependent. With Mg\(^{2+}\)
ADP + P_i in catalytic sites, the linkage of ε from the Cys at 108 is predominantly with Cys-381 of a β subunit, while in Mg^{2+} ATP (by using Mg^{2+} AMP-PNP) the ε subunit is linked to the equivalent position in the α subunit. The cross-linking results described here expand on our previous cryoelectron microscopy studies which had shown a shifting of the ε subunit by 20–25 Å between adjacent α and β subunits. In addition to interactions with α and β subunits, the ε subunit is also bound to the γ subunit (Dunn, 1982; Aggeler et al., 1992) and to the ε subunit ring of the FO part of the ATP synthase complex (Zhang and Fillingame, 1995). We suggest, therefore, that the movements of the ε subunit seen here, and in Wilkens and Capaldi (1994), are involved in energy coupling between the F_1 and F_0 parts.

Our data also show a nucleotide dependent movement of the γ subunit in concert with the movement of the ε subunit. The γ subunit can be cross-linked to a β subunit readily with ATP bound, less readily with Mg^{2+}-ADP bound in catalytic sites. In what may be its less tightly bound state, in Mg^{2+}-ADP, the γ subunit appears to be able to reside somewhat closer to an α subunit and can be trapped there by prolonged incubation in high concentrations of CuCl_2. This interaction between γ and β subunits involves Cys-87 based on the close positioning of this residue and β Glu-381 from the crystallographic data (Abrahams et al., 1994), and based on recent studies from Duncan et al. (1995) in which cross-linking between the β (DELS EED region) and γ subunit was abolished in the mutant ECF_1 β380Cγ/C87S. Cross-linking between the α and γ subunits most likely involves Cys-87 as well, but the possibility that Cys-112 is involved has not been ruled out.

In the mutant αS411G/βE381CαS108C, there is high yield cross-linking of α to β subunits by CuCl_2 treatment involving the Cys residues introduced into these subunits. This requires the rotation of the COOH-terminal α-helical region of α and/or β toward each other. That such a rotation can occur is evident in the recent high resolution structure of MF_1 (Abrahams et al., 1994). In the form of the enzyme crystallized, αε is arranged differently with respect to αTP or αTP in that the region involving Ser-411 is rotated so that it is very close to the region of βTP including E381, i.e. the distance between these two residues is 6.8 Å from α backbone carbon to α backbone carbon. The results presented here show that rotation of α subunits with respect to β subunits occurs not only in F_1, but also in F_0, implying that catalytic sites can be open at all times even in the presence of high concentrations of ATP or ADP.

In terms of the mechanism of cooperative ATP hydrolysis (and ATP synthesis) it is possible that the switching of the ε, and possibly the γ subunit, shown schematically in Fig. 5, is an oscillation between two adjacent subunit (pairs). If this switching is not back and forth, but continues clockwise or anti-clockwise, the rotation of the small subunit(s) could then “alternate” the different nucleotide affinities of the three catalytic sites.

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, 621–628
Aggeler, R., and Capaldi, R. A. (1993) J. Biol. Chem. 268, 14576–14578
Aggeler, R., Zhang, Y.-Z., and Capaldi, R. A. (1997) Biochemistry 36, 7107–7113
Aggeler, R., Chicas-Cruz, K., Cai, S.-X., Keana, J. F. W., and Capaldi, R. A. (1992) Biochemistry 31, 2956–2961
Aggeler, R., Cai, S. X., Keana, J. F. W., Kolke, T., and Capaldi, R. A. (1993) J. Biol. Chem. 268, 20831–20837
Aggeler, R., Haughton, M. A., and Capaldi, R. A. (1995) J. Biol. Chem. 270, 9185–9191
Beckers, G., Berzborn, R. J., and Strotmann, H. (1992) Biochim. Biophys. Acta 1101, 97–104
Boekema, E. J., and Böttcher, B. (1992) Biochim. Biophys. Acta 1098, 131–143
Boyer, P. D. (1993) Biochim. Biophys. Acta 1140, 215–220
Capaldi, R. A., Aggeler, R., Turina, P., and Wilkens, S. (1994) Trends Biochem. Sci. 19, 264–269
Capaldi, R. A., Aggeler, R., and Wilkens, S. (1995) Trans. Biochem. Soc. 23, 767–770
Dalimann, H. G., Flynn, T. G., and Dunn, S. D. (1992) J. Biol. Chem. 267, 18953–18960
Davis, L. G., Dibner, M. D., and Battey, J. F. (1986) Basic Methods in Molecular Biology, Elsevier Science Publishing Co., Inc., New York
Dunn, S. D. (1982) J. Biol. Chem. 257, 7354–7359
Gogol, E. P., Aggeler, R., and Capaldi, R. A. (1989a) Biochim. Biophys. Acta 1019, 4717–4724
Gogol, E. P., Lücken, U., and Capaldi, R. A. (1987) FEBS Lett. 219, 274–278
Gogol, E. P., Aggeler, R., Sagernann, 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
Kunkel, T. A., Roberts, J. D., and Zakour, M. A. (1987) Methods Enzymol. 154, 367–382
Laemmli, U. K. (1970) Nature 227, 680–685
Lötscher, H.-R., Jung, C., and Capaldi, R. A. (1984) Biochemistry 23, 4134–4140
Lücken, U., Gogol, E. P., and Capaldi, R. A. (1990) Biochemistry 29, 5339–5343
Malatis, J., Fritsch, E. F., and Sambrook, J. (1982) Molecular Cloning: A Laboratory Manual, Cold Spring Harbor Laboratory, Cold Spring Harbor, NY
Mendel-Hartvig, J., and Capaldi, R. A. (1991) Biochemistry 30, 1278–1284
Senior, A. E. (1990) Physiol. Rev. 68, 171–231
Senior, A. E. (1990) Annu. Rev. Biophys. Biophys. Chem. 19, 7–41
Turina, P., and Capaldi, R. A. (1994) J. Biol. Chem. 269, 13465–13471
Wilkens, S., and Capaldi, R. A. (1994) Biol. Chem. Hoppe-Seyler 375, 43–51
Zhang, Y., and Fillingame, R. H. (1995) J. Biol. Chem. 270, 24609–24614