Trapping of conformations of the *Escherichia coli* F_1 ATPase by disulfide bond formation

A state of the enzyme with all three catalytic sites of equal and low affinity for nucleotides

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Abstract A mutant of *Escherichia coli* F_1F_0-ATPase, αS411C/βY331W/βE381C/γC87S, has been generated. $CuCl_2$ treatment of this mutant led to cross-linking between α and β subunits in yields of up to 90%. This cross-linking across non-catalytic site interfaces inhibited ATP hydrolysis activity. In the absence of cross-linking, MgATP bound in catalytic sites of the mutant with three different affinities of 0.1 μM, 6 μM and 60 μM, respectively, values that are comparable to wild-type. For MgADP, there was one tight site (0.34 μM) and two sites of lower affinity (each 27 μM), again comparable to wild-type enzyme. After cross-linking all three catalytic sites bound MgATP or MgADP with the same relatively low affinity (~60 μM). Thus cross-linking fixed all three catalytic sites in the same conformation. Trypsin cleavage experiments showed that cross-linking fixed the ε subunit in the ATP+EDTA conformation.

Key words: F_1 Adenosine triphosphatase, cross-linking; Catalytic site nucleotide binding; ε Subunit conformation

1. Introduction

The ATP synthase F_1F_0 is a molecular motor which can reversibly catalyze the reaction ADP+P_i = ATP. The enzyme is simplest in bacteria such as *Escherichia coli* [1–3]. ECF_1F_0 is composed of a total of 8 different subunits, 5 constituting the F_1 part ($\alpha_3\beta_3\gamma_1$, δ_1, and ε_1), and three forming the F_0 part (a_1, b_2, c_{9-12}). There are three catalytic sites on the F_1 part, each located predominantly on β subunits, which appear to be linked during energy coupling to a single proton pore produced at the interface between the a subunit and the c subunit ring [4,5].

Beginning with cryoelectron microscopy studies [6], evidence has accumulated that each catalytic site is alternately linked with proton translocation via the rotation of a mobile domain constituted by the γ and ε subunits [7–10]. This mobile domain forms the 40–45-Å long stalk seen in electron micrographs [11]. A second stalk contributed by the δ and b subunits has been inferred, and this has been proposed to act as a stator, fixing the $\alpha_3\beta_3$ domain of F_1, possibly with the a subunit of the F_0 part [12,13]. In addition to rotating, the γ and ε subunits undergo conformational changes with each enzyme turnover that are an essential part of the coupling mechanism [14].

A recent X-ray structure of beef heart F_1 has visualized one state of the enzyme in which the 3 α–β pairs are each in a different conformation related to their different catalytic site occupancy [15]. Thus, one contains ADP and has a closed catalytic site, the second α–β pair contains AMP·PNP and is partly open, while in the third the catalytic site is empty and the interface between the two subunits is open. The arrangement of the γ subunit in this structure includes the interaction of a short α helix of residues 82–99 (*E. coli* sequence numbers) with the DELSEED region of that β subunit containing the AMP·PNP.

We have sought to stabilize conformers of ECF_1 and ECF_1F_0 by disulfide bond formation for study of their different structures and enzymatic properties. Mutants αS411C/εS108C and βE381C/εS108C have been described in which the γ and ε subunits can be cross-linked to α or β subunits, respectively [16,17]. This represents the fixing of the γ-ε rotor at positions offset by 60°. These two conformers had very different nucleotide binding properties [18,19]. We have also studied the mutant αS411C/βE381C/εS108C in which there is a nucleotide-dependent switching of the cross-linking of γ and ε subunits between α and β subunits [17]. In this mutant, a cross-link between the Cys at α-411 and β-381 was also obtained. Inspection of the X-ray structure of beef heart F_1 shows how this can be possible at one α-β interface. The C-terminal part of α_E is rotated toward β_{DP} such that the positions of α Ser-411 and β Glu-381 are close. Here, we have explored this cross-link in more detail. ECF_1 from the mutant αS411C/βY331W/βE381C/γC87S has been generated and cross-linked. Evidence is presented that all 3 α–β pairs can become cross-linked to yield a protein in which all three catalytic sites are of equal and low affinity for nucleotides.

2. Materials and methods

2.1. Construction of plasmids

The *unc* containing plasmid pRA163 with the mutations αS411C/βE381C/γC87S was created by ligating the 1-kbp *Bsu*36I fragment of pRA149 with the γC87S mutation [20] to the 6-kbp and 5.7-kbp *Bsu*36I fragments of pRA142 with the αS411C and βE381C mutations [17]. pRA164 was obtained by first ligating the 6.7-kbp *Eag*I fragment of pRA201 [18] with the 5.9-kbp *Eag*I fragment of pRA133 [16] producing pGG2, which contains the mutations βY331W/βE381C and then combining the 3-kbp *Sst*I/*Nsi*I fragment from this plasmid with the 9.7-kbp *Sst*I/*Nsi*I fragment of pRA163. Subcloning was carried

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out using XL1 Blue from Stratagene. *AN888* was the *unc*$^-$ strain used for transformation with pRA164.

2.2. Fluorescence measurements of the αS411C/βY331W/βE381C/γC87S ECF_1 mutant

The occupation of catalytic sites by nucleotides was detected directly by the tryptophan fluorescence of the mutation βY331W in a SLM 8000 photon counting spectrofluorometer at room temperature, according to Weber et al. [21] as modified by Grüber and Capaldi [18]. Calculations of kinetic constants were performed using the KaleidaGraph data analysis and graphics program for personal computers.

2.3. Other methods

ATPase was isolated [22,23] and cross-linked with $CuCl_2$ after precipitation with 70% ammonium sulfate and two consecutive centrifuge columns (Sephadex G50 medium), which were equilibrated in 50 mM MOPS, pH 7.0, 0.5 mM EDTA and 10% glycerol as described in Aggeler et al. [16]. Proteolytic cleavage of ECF_1 was carried out, with or without prior cross-linking with $CuCl_2$, in 50 mM Tris, pH 8.0, 0.5 mM EDTA and 10% glycerol at room temperature at a protein concentration of 0.5 mg/ml, with trypsin at a ratio of 1:50 (w/w) as described by Mendel-Hartvig and Capaldi [24]. SDS-polyacrylamide gel electrophoresis was carried out according to Laemmli [25]. ATPase hydrolysis activity was determined with an ATP regenerating system [26] and protein concentrations with the BCA protein assay from Pierce.

3. Results

The mutant used in this study is αS411C/βY331W/βE381C/γC87S. It contains the Cys at α 411 and β 381, the effect of cross-linking of which is to be examined. The intrinsic Cys of the γ subunit (Cys-87) has been replaced by a Ser to avoid competition of cross-linking of the α or β site to the γ subunit.

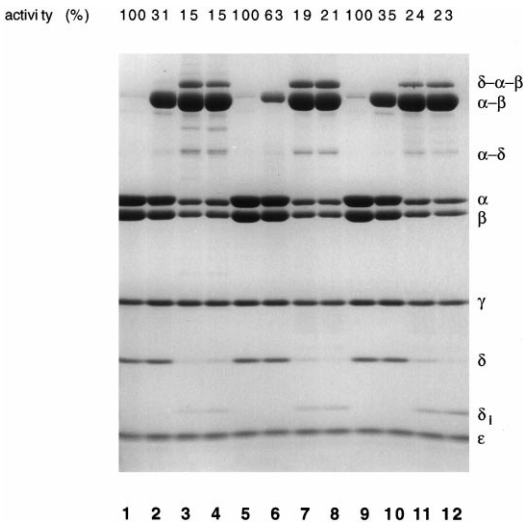

Fig. 1. $CuCl_2$ induced cross-linking of ECF_1 from αS411C/βY331W/βE381C/γC87S. F_1 ATPase was obtained after column centrifugation at 0.25 mg/ml in 50 mM MOPS, pH 7.0, 0.5 mM EDTA and 10% glycerol and was supplemented with 2.5 mM $MgCl_2$ (lanes 1–4), 2.5 mM $MgCl_2$+2 mM ATP (lanes 5–8) and 2.5 mM $MgCl_2$+2 mM NaN_3+2 mM ATP, respectively. Cross-linking was carried out for one hour at room temperature by adding $CuCl_2$ at 5 μM (lanes 2, 6 and 10), 60 μM (lanes 3, 7 and 11), and 150 μM (lanes 4, 8 and 12). 7.5 mM EDTA was added and ATPase hydrolysis activity determined as 11.4, 9.8 and 7.6 μmol ATP hydrolyzed per min per mg protein for the control samples without $CuCl_2$ in lanes 1, 5 and 9, respectively. After addition of 20 mM *N*-ethylmaleimide and dissociation buffer without reducing agent, 21 μg protein were applied per lane on a 10–18% polyacrylamide gel. Coomassie brilliant blue was used for staining.

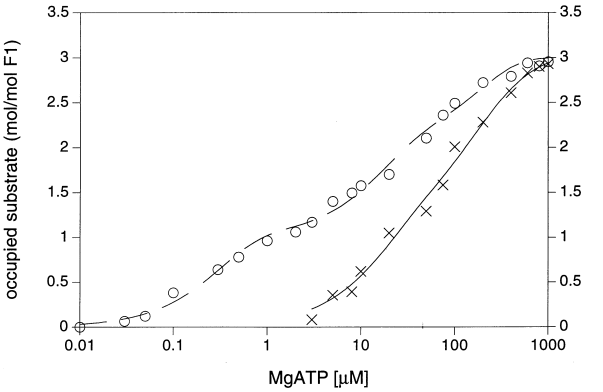

Fig. 2. MgATP binding to the mutant ECF_1 (αS411C/βY331W/βE381C/γC87S) ECF_1 mutant (100 nM), from which nucleotides had been removed, was diluted in a buffer containing 50 mM MOPS, pH 7.0 and 10% glycerol (v/v) and titrated with MgATP (1:1) to a volume of 400 μl in a 0.4×1-cm quartz cuvette. The nucleotide binding stoichiometries were calculated from the decrease in fluorescence of residue βW331, which was recorded at $\lambda_{em} = 342$ nm and $\lambda_{ex} = 285$ nm. The measurements were carried out in an SLM 8000 photon counting spectrofluorometer with the emission slit at 8 nm. ○, αS411C/βY331W/βE381C/γC87S mutant; ×, αS411C/βY331W/βE381C/γC87S mutant after cross-linking with 100 μM $CuCl_2$. The lines represent theoretical binding curves based on the K_d values given in Table 1.

The presence of a Trp for Tyr at position 331 of β allows a direct measurement of nucleotide binding (see later). ECF_1 isolated from this mutant showed an ATPase activity in the same range as wild-type enzyme preparations, i.e. 11 μmol ATP hydrolyzed per min per mg protein.

3.1. Cross-linking of α to β subunits

Fig. 1 shows $CuCl_2$-induced cross-linking in this mutant over a range of Cu^{2+} concentrations and under different nucleotide conditions. The predominant cross-linked product in all cases is between α and β, involving α Cys-411 and β Cys-381. A fraction of the cross-linked α–β pairs also have the δ subunit cross-linked to them via a disulfide bond from the intrinsic Cys-140 of δ and the intrinsic Cys-90 of the α subunit. Small amounts of α–δ product as well as internally cross-linked δ (δ_i) were also resolved. A nucleotide dependence of the α–β cross-linking is clearly evident at 5 μM $CuCl_2$. ADP+Mg^{2+} in catalytic sites reduces the efficiency of the cross-linking.

Based on the disappearance of α or β, the yield of cross-linking routinely reached between 80 and 90%. This can only occur if all three α–β pairs have been cross-linked in most molecules. There was residual ATPase activity, which was higher than expected for the yield of cross-linking (see Fig. 1). This residual ATPase activity was inhibited by dicyclohexylcarbodiimide, a reagent that blocks cooperativity and thereby lowers multisite ATPase activity without affecting unisite catalysis significantly [27]. Therefore, the unexpectedly high ATPase activity is not due to enhanced unisite catalysis (in all three sites at once).

It seems likely that covalent cross-linking of any one of the three α–β pairs in any F_1 molecule should impair alternation of catalytic sites, and therefore reduce cooperative activity, if the enzyme requires all three sites to be operational. Therefore, the activity data on cross-linked enzyme could represent residual relatively efficient bi-site catalysis or, for some reason,

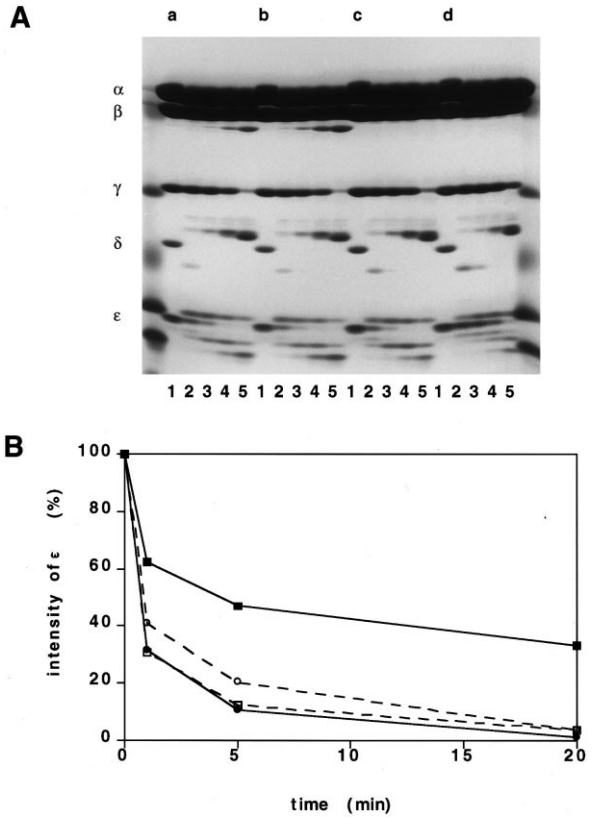

Fig. 3. Trypsin cleavage of cross-linked and non-cross-linked ECF_1 from αS411C/βY331W/βE381C/γC87S. A: ATPase was cross-linked at 0.7 mg/ml with 150 μM $CuCl_2$ after addition of 2.5 mM $MgCl_2$ and 2 mM ATP. After incubation for one hour at room temperature 5 mM EDTA was added. Nucleotides were removed by ammonium sulfate precipitation and two centrifuge columns in 50 mM Tris, pH 8.0, 0.5 mM EDTA and 10% glycerol. Trypsin was added to the ATPase at 0.5 mg/ml after supplementing with 2 mM ATP (panel a) and 2 mM ATP+2.5 mM $MgCl_2$ (panel b), respectively. 80-μl samples were withdrawn and added to 1 μl 0.3 M PMSF after 1 min (lanes 2), 5 min (lanes 3), 20 min (lanes 4) and 80 min (lanes 5); no trypsin was added to control samples (lanes 1). Trypsin cleavage of non-cross-linked ATPase is shown in panel c (addition of ATP) and panel d (addition of $MgCl_2$+ATP). Samples containing 30 μg ATPase were loaded on a 10–18% gel after dissociation in the presence of 50 mM dithiothreitol. Molecular weight markers were applied on a lane on the left and right. B: The disappearance of the ε-subunit was quantitated by scanning the Coomassie Brilliant Blue-stained gel with an Epson Action Scanner II and analyzing the intensity of the protein bands with NIH Image 1.60. Open symbols: cross-linked ATPase; filled symbols: non-cross-linked ATPase; circles: EDTA+ATP; squares: $MgCl_2$+ATP.

there could be a significant fraction of enzyme molecules that have no cross-linking. It is not possible to distinguish easily between these possibilities.

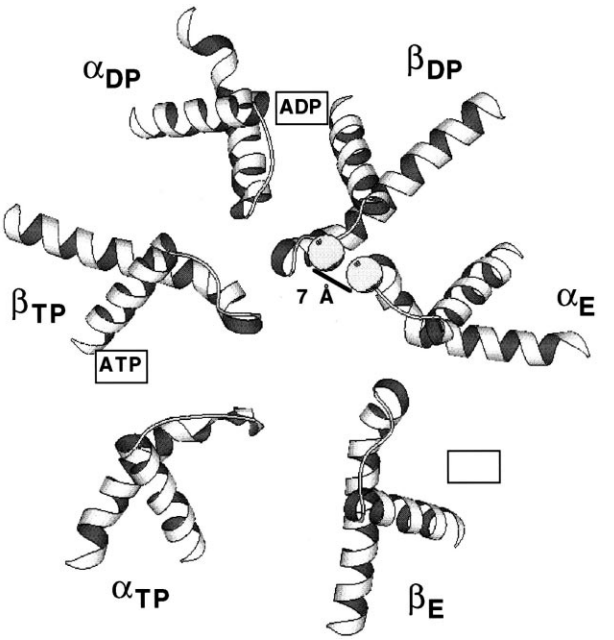

Fig. 4. View of DELSEED area of F_1 ATPase from membrane side. Structure of MF_1 from residues 385–427 of α and 365–413 of β according to the coordinates [15]. The spheres indicate the positions of α_ES411C and β_{DP}E381C in ECF_1, respectively. Squares are placed in catalytic-site interfaces.

3.2. Nucleotide binding in catalytic sites

By including the mutation βY331W it was possible to follow nucleotide binding into the mutant, both before and after cross-linking, by monitoring the fluorescence change of the introduced Trp residue. Fig. 2 plots the data for binding of ATP to the mutant in the presence of Mg^{2+}. Non-treated enzyme showed three binding sites for ATP with the first and third sites differing in binding affinities by at least 600-fold (Table 1). The data obtained after cross-linking was best fitted by binding of 3 mol of ATP, all with the same affinity of 60 μM. The affinities of the three catalytic sites for ADP+Mg^{2+} were also measured and are listed in Table 1. As with ATP, there was a strong cooperativity in binding of nucleotides before cross-linking which was lost in the cross-linked enzyme.

3.3. Effect of α-β cross-linking on the arrangement of the ε subunit

We have established previously that there is a nucleotide dependence of the interaction of the ε subunit with the core ECF_1 complex [17,24]. In the presence of ADP+Mg^{2+} and with P_i present, the ε subunit is in a different arrangement than when ATP is bound (e.g. as ATP+EDTA or AMP·PNP+Mg^{2+}). In the ADP state, the ε subunit is readily cross-linked from the C-terminal domain to a β subunit and is

Table 1
Binding parameters of nucleotides of the αS411C/βY331W/βE381C/γC87S ECF_1 mutant as determined by fluorescence signal of βW331

Ligand	Preparation	K_{d1} (μM)	K_{d2} (μM)	K_{d3} (μM)
MgATP	ECF_1 mutant	0.1	6.0	61
MgATP	α-β-δ cross-linked ECF_1 mutant	60	60	60
MgADP	ECF_1 mutant	0.34	27	27
MgADP	α-β-δ cross-linked ECF_1 mutant	63	63	63

protected from protease digestion, while in the ATP state cross-linking to β is less readily achieved (because of movements of the C-terminal domain towards α), and the ε subunit becomes sensitive to trypsin digestion. Fig. 3 shows the profile of trypsin cleavage in ATP+EDTA and ADP+Mg^{2+}+P_i before and after cross-linking.

The SDS-polyacrylamide gel (Fig. 3A) shows a rapid disappearance of ε into cleavage products in ATP+EDTA, but a slow cleavage in ADP+Mg^{2+} for the non-cross-linked enzyme. After cross-linking, ADP+Mg^{2+} fails to protect the ε subunit from trypsin cleavage. This difference is clear in Fig. 3B, which shows the rate of disappearance of intact ε subunit under the different conditions.

4. Discussion

The F_1F_0 type ATPase is a highly dynamic enzyme that must cycle through many conformations during ATP hydrolysis-linked proton translocation and, in the reverse direction, during proton gradient driven ATP synthesis. We are trying to trap a range of conformations of this enzyme by stabilizing them via disulfide bond formation between subunits. We had previously found that a disulfide bond can be formed between α and β subunits via a Cys at position 411 of α and a Cys at position 381 of β [17]. Based on the X-ray data for the beef heart enzyme, these sites are at equivalent positions in a loop of the relatively conserved C-terminal domains of α and β, respectively. In the conformation of the enzyme observed by Abrahams et al. [15], the C-terminal domain of one of the three α subunits (α_E) is rotated around so that it comes close to the DELSEED region of the next β subunit (β_{DP}) and in disulfide bond-forming distance (Fig. 4). This occurs across a non-catalytic site interface and, by the movement of α and the rotation away of the C-terminal domain of β_E, one catalytic site is open and is empty of nucleotide.

Here, we show that the enzyme can be trapped in a conformation in which all three α and β subunits interact via their C-terminal domains across the non-catalytic site interfaces. The nucleotide binding affinity of the catalytic sites was measured after cross-linking by using the fluorescence change of the Trp mutated into these sites. The binding constant is around 60 μM with little or no discrimination for ADP or ATP. An affinity in this range is seen for the third and lowest affinity site in wild-type or non-cross-linked mutant ECF_1. The rotation of the C-terminal domain breaks the catalytic site by swinging the P loop region away from the adenine binding pocket. The nucleotide binding we observe, therefore, probably represents binding at the adenine pocket, explaining the similarity of binding of ADP and ATP at this site. Based on nucleotide binding properties, the conformation stabilized here is the same as obtained for ECF_1 in the presence of nucleotides but without Mg^{2+}. Thus, the enzyme binds 3 mol of ATP with an equal and low affinity (60–80 μM) in the absence of Mg^{2+}, i.e. the so-called ATP/EDTA state [18,28].

As described previously [17,24,29] and shown here, the ε subunit is in a different arrangement with ADP/Mg^{2+} bound in catalytic sites than with ATP/EDTA present. In enzyme cross-linked across non-catalytic site interfaces, the ε subunit remains in its ATP/EDTA state even when ADP/Mg^{2+} is bound. The ability to trap conformations such as the one described here, along with states in which the catalytic sites are non-equivalent, e.g. with γ and ε subunits linked to α or β subunits, offers important possibilities. We have recently obtained crystals of ECF_1 which diffract to higher than 6.5 Å resolution [30]. Preliminary studies show that cross-linked enzyme preparations also crystallize, offering the possibility that details of the arrangement of subunits in the different conformers can be worked out.

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