The coupling region of F_0F_1 ATP synthase: binding of the hydrophilic loop of F_0 subunit *c* to F_1

T. Licher, E. Kellner, H. Lill*

Abteilung Biophysik, Universität Osnabrück, D-49069 Osnabrück, Germany

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Abstract The hydrophilic loop region of the CF_0 *c* subunit has been expressed as a fusion with MalE in *Escherichia coli*. A cysteine was introduced at the C-terminus to allow fluorophore labeling of the fusion protein. After removal of the MalE moiety, the labeled peptide was used for binding studies with fluorophore-labeled CF_1. At saturation, 1 mol peptide was bound per mol CF_1. Binding was abolished after removal of subunit ε from CF_1, and partially restored by addition of recombinant ε.

Key words: ATP synthase; ATPase; F_0F_1; Proteolipid; Coupling

1. Introduction

F_0F_1 ATP synthase, the central enzyme in chemiosmotic energy supply, comprises two subcomplexes: the F_0 part allows transmembrane transfer of coupling ions, and F_1 harbors the nucleotide binding sites of the enzyme. In *Escherichia coli*, F_0 is assumed to contain subunits *a*, *b*, and *c* in a 1:2:12 stoichiometry. The 8 kDa *c* subunit contains two membrane-spanning helices, connected by a short hydrophilic loop. These proteins, also named proteolipids, are directly involved in ion transfer through F_0 [1]. The F_1 part contains subunits α, β, γ, δ, and ε in a 3:3:1:1:1 stoichiometry. A large part of the structure of mitochondrial F_1 has been solved by X-ray crystallography [2]. The nucleotide binding sites of the enzyme are located on interfaces of the large α and β subunits, which are arranged alternately in a hexagonal structure. Parts of the γ subunit form two coiled helices in the center of this hexagon. The γ subunit rotates during F_1-mediated ATP hydrolysis [3–5], probably in conjunction with ε [6,7].

Transfer of ions through F_0 is coupled to hydrolysis or synthesis of ATP in F_1. The mechanism of the coupling of events in both parts of the enzyme has only recently been emerging. Models under consideration suggest a ring of *c* subunits, which comprises an extension of the rotor element from F_1 into F_0, rotating relative to an *ab* assembly. A stator element, made up from F_1 δ and F_0 *ab*, is proposed to prevent concomitant rotation of the αβ hexagon [8]. Some hydrophilic loop regions of *c* subunits in F_0 are located in close proximity to the γ and ε subunits in F_1 [9,10], and interactions between proteolipids and F_1 have been shown [11]. We have now expressed the hydrophilic part of the *c* subunit as a C-terminal fusion with MalE. After removal of the MalE part, the soluble peptide (c_{sol}) was used to study interactions with F_1.

2. Materials and methods

The vector pMAL-c2 (New England Biolabs) was digested with endonucleases *Xmn*I and *Eco*RI. A pair of synthetic oligonucleotides was introduced, yielding a C-terminal extension of 20 additional amino acids as detailed in Fig. 1. The sequence was taken from the hydrophilic part of the *c* subunit (c_{sol}) of the cyanobacterium *Synechocystis* sp. PCC 6803 [12], with an additional cysteine residue introduced at the C-terminus.

After verification by DNA sequencing, the fusion protein was expressed in *E. coli* DH5α (BRL Life Technologies). If not otherwise stated, all following procedures were carried out on ice, and centrifugation was performed at 4°C. After 2 h of induction, cells were harvested by 20 min centrifugation at 4000×*g* and resuspended in 10 ml column buffer per gram of cells (wet weight, column buffer: 20 mM Tris-HCl, pH 7.4, 1 mM EDTA, 300 mM NaCl). After addition of 50 μM PefablocSC (Boehringer) and 150 U Benzonase (Merck), cells were sonicated (2 min in a Branson sonifier, 70% output, 50% pulse on ice) and centrifuged for 20 min at 9000×*g* and another 30 min at 100 000×*g*. The supernatant was diluted to 2.5 mg/ml protein by addition of column buffer and loaded on a disposable column, containing 4 ml amylose resin (New England Biolabs). The column was washed with 12 volumes of column buffer and then eluted with up to 10 ml of column buffer containing 10 mM maltose. The purified fusion protein was precipitated in 90% (w/v) $(NH_4)_2SO_4$.

Prior to labeling, the precipitated protein was pelleted for 15 min at 100 000×*g* and resuspended in 500 μl buffer, containing 50 mM NaCl, 2 mM EDTA, 50 mM Tris-HCl, pH 7.0 (TNE7 as in [13]). Buffer exchange was by gel filtration over TNE7-equilibrated Sephadex G50 (Pharmacia) and, after addition of 1 mM dithiothreitol (DTT), the protein was incubated for 1 h at room temperature. Following another gel filtration against TNE7 to remove DTT, fluorescein-5-maleimide (fluorescein) was added in 60 times molar excess and the labeling mix was incubated for 2 h at room temperature. The protein was then precipitated in 90% (w/v) $(NH_4)_2SO_4$. A fraction was resuspended to spectroscopically determine the labeling stoichiometry, which was routinely more than 0.9 mol fluorescein per mol fusion protein.

Prior to protease digestion, the precipitated protein was pelleted for 15 min at 100 000×*g* and resuspended at 2.5 mg/ml in buffer containing 2 mM $CaCl_2$, 100 mM NaCl, 20 mM Tris-HCl, pH 8.0 (Xa buffer). Factor Xa (New England Biolabs) was added to a final ratio of 2% (w/w) of total protein, and the sample was incubated for 24 h at room temperature. The mixture was then passed through an ultrafiltration unit with 5 kDa exclusion size (Millipore). Whereas factor Xa and MalE were retained by the membrane, the labeled peptide was found in the filtrate, and its concentration was determined by fluorescein absorption.

Preparation of CF_1 and $CF_1(-\delta\varepsilon)$ and labeling of subunit γ with 7-diethylamino-3-(4′-maleimidylphenyl)-4-methylcoumarin (coumarin) was carried out as described previously [14]. For energy transfer measurements, a stock solution of labeled enzyme at 100 nM was prepared in TNE7. Aliquots of this stock were supplemented with identical volumes of various concentrations of labeled peptide in TNE7, yield-

*Corresponding author. Fax: (49) (541) 969 2870.
E-mail: lill@uni-osnabrueck.de

Abbreviations: CF_1, spinach chloroplast F_1 ATPase; $CF_1(-\delta\varepsilon)$, CF_1 lacking subunits δ and ε; Coumarin, 7-diethylamino-3-(4′-maleimidylphenyl)-4-methylcoumarin; Fluorescein, fluorescein-5-maleimide; FRET, fluorescence resonance energy transfer; PCR, polymerase chain reaction; SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis; Tris, *N*-tris-(hydroxymethyl)-aminomethane

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GGT CAA GCC GTT TCT GGT ATT GCG CGC CAG CCT
CCA GTT CGG CAA AGA CCA TAA CGC GCG GTC GGA

 G   Q   A   V   S   G   I   A   R   Q   P


GAA GCT GAA GGA AAA ATT CGG GGT TGC T
CTT CGA CTT CCT TTT TAA GCC CCA ACG ATT AA

 E   A   E   G   K   I   R   G   C   •
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Fig. 1. Oligonucleotides used for the construction of the fusion protein. Two oligonucleotides have been constructed which encode the following features: a blunt end; a 19 amino acid peptide (c_{sol}) comprising the hydrophilic loop region of the *Synechocystis* sp. PCC 6803 *c* subunit (typed boldfaced); a cysteine at position 20 of the peptide; the stop codon overlapping the staggered ends of an *Eco*RI site.

ing several samples with 80 nM F_1 and ratios of enzyme:peptide ranging from 1:0 to 1:50.

Coumarin fluorescence was measured as described [14]. Assuming one binding site per enzyme, the observed quenching can be attributed to the stoichiometry of labeled peptide bound per enzyme (cf. [15]):

$$Q = \frac{Q_m}{2} \cdot \left[\left(1 + \frac{[L]}{[E]} + \frac{k_D}{[E]}\right) - \sqrt{\left(1 + \frac{[L]}{[E]} + \frac{k_D}{[E]}\right)^2 - 4 \cdot \frac{[L]}{[E]}} \right] \quad (1)$$

where Q is the observed quenching, Q_m is the maximal quenching at saturated binding, $[E]$ is the total concentration of enzyme, $[L]$ is the concentration of the ligand, and k_D the dissociation constant.

3. Results

As shown in panel A of Fig. 2, large amounts of protein were found in the insoluble as well as in the soluble fractions of the cells (Fig. 2, lanes a and b) after expression of the MalE-c_{sol} fusion in *E. coli*. From the soluble material, the fusion protein could be easily purified by affinity chromatography on amylose resin (lane c). Cleavage by factor Xa (lane d) and subsequent ultrafiltration at 5 kDa exclusion limit yielded c_{sol} peptide completely devoid of any impurities (lane e). After incubation of the fusion protein with fluorescein, gel electrophoresis (Fig. 2B) revealed that the label was solely incorporated into the fusion protein (lane f). Protease treatment released the label from the fusion protein (lane g), and the labeled peptide was free from fluorescent impurities after ultrafiltration (lane h).

This fluorescein-labeled c_{sol} was then used in binding studies with spinach CF_1. The enzyme was coumarin-labeled at the regulatory disulfide bridge in the γ subunit [15]. Samples with identical concentrations of enzyme were mixed with increasing concentrations of labeled peptide and fluorescence spectra were recorded, showing a pronounced quenching of the coumarin fluorescence dependent on the amount of added peptide (Fig. 3). Quantification of the spectral data (Fig. 4A, open squares) revealed an initial steep decrease of fluorescence, followed by a more linear course. In contrast, upon using $CF_1(-\delta\varepsilon)$ in the binding assay, the steep decrease was completely lost and only the linear portion of the quenching was retained (Fig. 4A, open circles). Whereas we assume that the initial quenching was due to binding of c_{sol} to CF_1, the linear loss of fluorescence was attributed to some effect other than resonance energy transfer. We therefore fitted the data obtained with $CF_1(-\delta\varepsilon)$ to a straight line and corrected the data obtained with CF_1 accordingly. These data (closed squares in Fig. 4A) were then fitted by non-linear least squares analysis to Eq. 1, generating the binding curve shown in Fig. 4A. Finally, we attempted to examine the inability of $CF_1(-\delta\varepsilon)$ to bind c_{sol}. Purified recombinant subunit ε at a concentration found earlier to saturate inhibition of Ca-

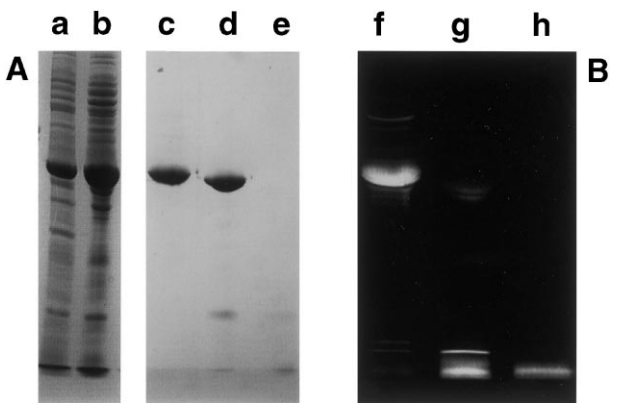

Fig. 2. SDS-PAGE of the MalE-c_{sol} fusion. A: Coomassie-stained SDS-PAGE gel of the following samples: lane a: insoluble material of *E. coli* cells after expression of the fusion protein; lane b: soluble fraction of the same cells; lane c: fusion protein after affinity chromatography on amylose resin and fluorescein incubation; lane d: the same fraction as in lane c after factor Xa digestion; lane e: the c_{sol} peptide after ultrafiltration. B: Fluorescence: lane f: purified and labeled sample as in lane c; lane g: protease digested sample as in lane d; lane h: labeled c_{sol} after ultrafiltration.

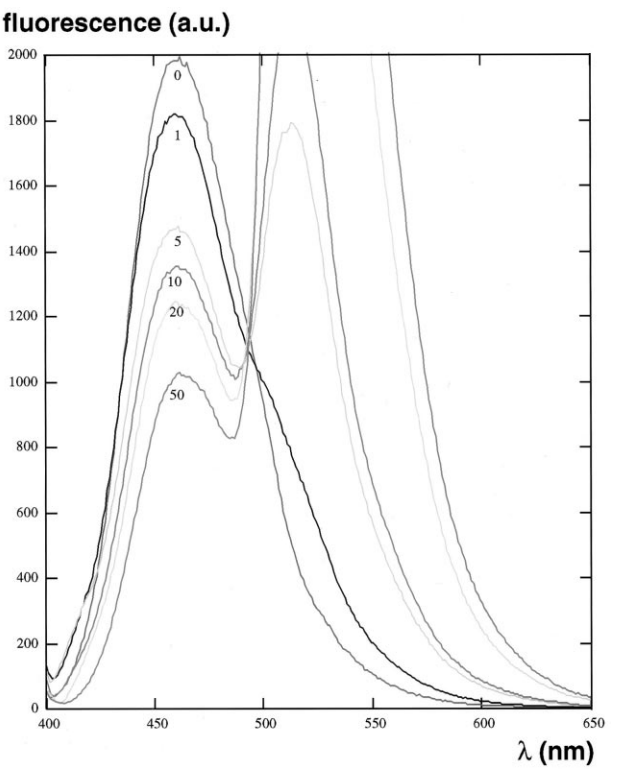

Fig. 3. Titration of coumarin-labeled CF_1 with fluorescein-labeled c_{sol}. Coumarin-labeled CF_1 was dissolved in Xa buffer. Aliquots of 140 μl were prepared in the same buffer, containing 80 nM CF_1 and indicated concentrations of fluorescein-labeled c_{sol}. After 1 h incubation at room temperature in the dark, the fluorescence emission under excitation at 397 nm was recorded.

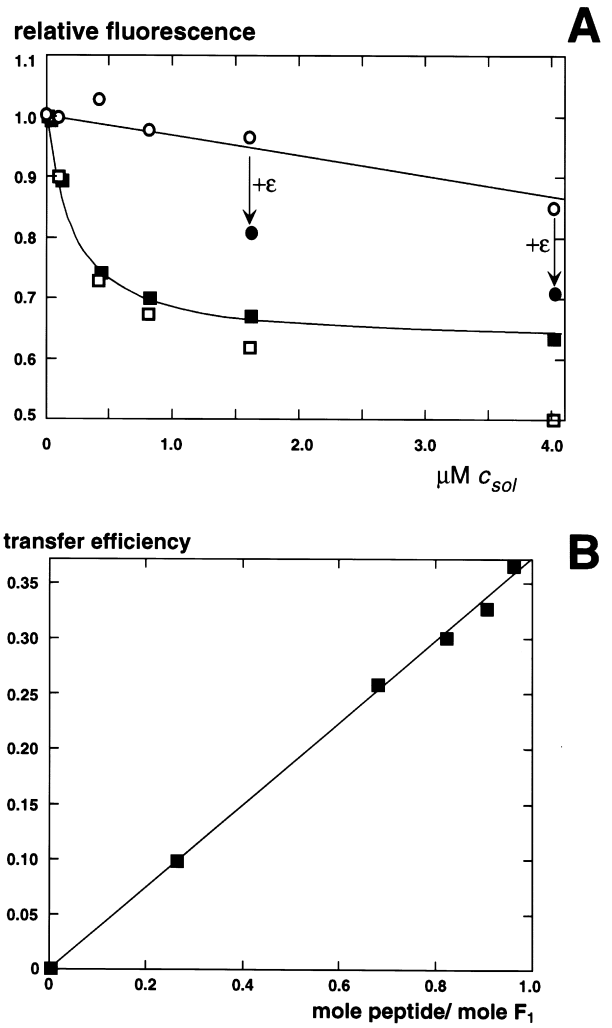

Fig. 4. Binding of c_{sol} to CF_1 and $CF_1(-\delta\varepsilon)$ as monitored by fluorescence resonance energy transfer. Data as depicted in Fig. 3 have been quantified in terms of quenching of coumarin fluorescence (panel A: open squares, or, after subtraction of linear quenching, filled squares) or as energy transfer efficiencies (panel B). The same measurements were done with $CF_1(-\delta\varepsilon)$ (open circles). At two c_{sol} concentrations with $CF_1(-\delta\varepsilon)$, arrows and filled circles depict the effect of the addition of 33 μM recombinant ε subunit.

ATPase on $CF_1(-\varepsilon)$ [16] was added to the binding assay. As a result, we observed substantial restoration of fluorescence quenching (Fig. 4A, filled circles). This finding may explain an observation made in the course of this work: the saturation of binding occurred with different CF_1 preparations at different stoichiometries, ranging in gel filtration assays from 0.4 to 0.9 mol c_{sol} bound per mol CF_1 (not shown). We tentatively attribute this effect to different amounts of ε being present in the various preparations, since it is known that the small subunits can be easily lost during CF_1 purification. As a consequence, whereas the fit shown in Fig. 4A yielded a k_D of 160 nM, in other experiments k_D values of up to 1.2 μM have been found.

Eq. 1 is based on the assumption of one binding site being present per enzyme. We scrutinized this assumption by a transformation of the quenching data into energy transfer efficiencies ($E = 1-Q$). The k_D obtained by the fit to Eq. 1 was used to calculate binding stoichiometries in mol c_{sol} per mol enzyme. The resulting data were then replotted as shown in Fig. 4B. As described by Shapiro et al. [13], with one binding site per enzyme energy transfer is directly proportional to binding stoichiometry. The presence of an additional binding site on the enzyme would cause substantial deviation from linearity at higher concentrations of ligand, which were clearly not observed here.

4. Discussion

The new classification of F_0F_1 subunits into parts of stator and rotor, respectively, implies more than one region of interaction between the two parts of the enzyme. Very recently, the proposed 'second stalk' has become evident from electron micrographs [17]. In addition, recent findings indicate that subunits γ and ε are in very close proximity to the hydrophilic loop region of subunit *c* [9,10]. Whereas these studies merely showed spatial relationship of subunits, the conclusion that this was a region of interaction between F_0 and F_1 appeared reasonable, since tight interaction of the proteolipid with CF_1 has also been found [11]. Our findings now directly demonstrate interaction of the hydrophilic loop region of *c*, presumably with subunit ε. We have no indication that subunit γ is involved in this binding, although a concerted binding to γ and ε remains conceivable. Since we have expressed both recombinant F_1 subunits in *E. coli* [18], we can now examine binding of c_{sol} to these proteins alone or in concert.

Currently, there are no experimental data available concerning the mode of propagation of energy from F_0 to F_1 and vice versa. With isolated proteolipids, the deprotonation of the aspartic acid positioned in one transmembrane helix and identified as essential in ion conduction through F_0 gave rise to pronounced structural changes in the loop region [19]. The loop could push against γ and/or ε and propel their rotation. Such a mechanism would not require binding of the *c* subunit to F_1, which would even be detrimental. It also fails to account for the high asymmetry of subunits found in F_0, with one subunit *a* and up to 12 subunits *c* being involved in ion conduction [20]. The model developed by Junge in analogy to the flagellar motor [8] envisions rotation of a ring of *c* subunits as a result of ion conduction through F_0, driving a concomitant rotation of γ and ε in F_1. Such a mechanism requires tight interactions of proteolipids with F_1. The question remains whether binding of only one proteolipid to F_1, as found in this study, may confer the binding energy sufficient to sustain the torque generated in the rotor. According to direct observations of rotation by Yoshida and coworkers, the torque exceeds 40 pN nm under load [5]. Given a rotation of the rotor by 120° per ATP molecule, this adds up to about 50 kJ/mol, with a markedly higher starting torque. With only a few polar residues present in the loop region of *c*, those participating directly in binding still await identification. In any case, one expects only few salt bridges between *c* and F_1, each contributing between 80 kJ/mol (at a dielectric constant of 4 as in membranes) and 4 kJ/mol (with $\varepsilon = 80$ as in water). From these considerations, it might be necessary, at least in active enzymes, to have more proteolipids bound to F_1. We are currently investigating this possibility by binding studies with activated enzymes.

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