Site-directed mutagenesis of two conserved charged amino acids in the N-terminal region of α subunit of *E. coli*-F_0F_1

Barbara Hase[a], Sabine Werner-Grüne[a], Gabriele Deckers-Hebestreit[b], Heinrich Strotmann[a,*]

[a]*Institut für Biochemie der Pflanzen, Heinrich Heine Universität Düsseldorf, Universitätsstr. 1, 40225 Düsseldorf, Germany*
[b]*Fachbereich Biologie/Chemie, Arbeitsgruppe Mikrobiologie, Universität Osnabrück, Osnabrück, Germany*

Received 11 January 1996; revised version received 26 January 1996

Abstract Two conserved charged amino acids of the N-terminal 'crown' region of the α subunit of *E. coli*-F_1, α-D36 and α-R40 were exchanged for chemically related (α-D36→E, α-R40→K) or unrelated amino acids (α-D36→K, α-R40→G), respectively, by employing oligonucleotide-directed mutagenesis. ATP formation and ATP hydrolyzing activity of isolated plasma membrane vesicles was strongly inhibited in mutant HS2 (α-D36→K), but only slightly affected in the other mutants. The inhibition is not due to a lower content of F_0F_1 in HS2. In this mutant the extent of the proton gradient generated by ATP hydrolysis was more than 80% inhibited; in all other transformants much smaller effects were observed. The proton gradient established by NADH oxidation was 33% decreased in HS2, but was decreased to a lesser extent in all other mutants. After blockage of F_0 by DCCD treatment, the same NADH-induced proton gradient was obtained in all transformants including HS2. This and the fact that the activity of NADH oxidation was unchanged indicate increased proton leakiness of F_0F_1 carrying the α-D36→K mutation. In F_1 α-D36 is located in a domain contacting the β subunit in the vicinity of the arginine β-R52. The effect of α-D36→K replacement on catalysis and coupling thus may be due to an electrostatic repulsive effect in the crown region which alters the α and β subunit interaction.

Key words: *E. coli*; F_0F_1; α Subunit; H^+-ATPase; Site-directed mutagenesis

1. Introduction

F_0F_1-type H^+-translocating ATPases ('ATP synthases') catalyze the formation of ATP from ADP and phosphate in oxidative phosphorylation or photophosphorylation driven by a transmembrane electrochemical potential difference of protons, which is generated by electron transport. It is likely that energy transduction involves conformational changes of F_0F_1. In the so-called 'energy-linked binding change' hypothesis, the binding of the substrates ADP and phosphate and/or the release of the product ATP are considered to be driven by the proton gradient while ATP formation in the catalytic site is an isoenergetic reaction [1].

The ATP synthases which are structurally and functionally similar in bacteria, mitochondria and chloroplasts, consist of two subcomplexes, the membrane-integral F_0 sector which is responsible for proton translocation and the catalytic extrinsic F_1 sector. The F_0 sector of *E. coli* consists of three different subunits a, b and c in a stoichiometry of $a_1b_2c_{10\pm1}$, F_1 is composed of five different subunits α to ε in a stoichiometry of $\alpha_3\beta_3\gamma_1\delta_1\varepsilon_1$ [2]. The genes are located in one operon, the so-called *atp* operon [3].

X-ray analysis of crystals of F_1 from beef heart mitochondria at a resolution of 2.8 Å [4] shows the three α and β subunits alternately arranged like the slices of an orange and demonstrates the location of the catalytic and non-catalytic nucleotide binding sites on the β and α subunits, respectively. The N-terminal ends of the α and β subunits form a so-called 'crown' consisting of linked barrel structures. The 3D structures of α and β including the barrel structures of the crown are almost identical. The crown is thought to keep the three α and three β subunits together by hydrogen bonds between the polypeptide backbones [4]. The helical N- and C-terminal ends of subunit γ reach into the central cavity formed by the α and β subunits. The two helices protrude at the pole which is thought to form the connection to F_0. A large part of subunit γ as well as the smaller F_1 subunits are invisible in the crystal structure. They probably participate in the formation of the stalk which connects F_1 with F_0.

The 3D structure of F_1 permits precise design of mutagenesis experiments to investigate the role of a specific structure for function. Here we are interested in the functional role of the N-terminal ends of α and β subunits which form the mentioned crown structure. There are two clusters of highly conserved amino acids in α subunit, one in positions 36–44 and another in positions 65–74 (*E. coli*). They constitute the strands which form the connections to the corresponding strands of the β subunits which are less conserved. Among the identical amino acids of the 36–44 peptide in α there are the two charged amino acids α-D36 and α-R40. In the 3D structure α-D36 may be located close to the conserved basic amino acid β-R59 of β subunit. This pair is of particular interest because a possible ionic interaction between the two amino acids might stabilize the crown, at least we might expect an electrostatic destabilization of the crown if α-D36 is exchanged for a basic amino acid. Here we have replaced by site-specific mutagenesis the negatively charged aspartate α-D36 by the oppositely charged lysine (α-D36→K) and by the related glutamate (α-D36→E), respectively. Moreover, we have exchanged the basic amino acid α-R40 which has no charged counterpart on β subunit. The effect of the mutations on the functional properties of F_0F_1 was investigated.

2. Materials and methods

2.1. Mutagenesis

For mutagenesis the *atpA*-deficient *E. coli* strain RH304 [5] was employed as acceptor and the plasmid pDP34 [6], carrying the *atp* operon, was used as donor for complementation. To make the plasmid suitable for mutagenesis in *atpA*, pDP34 was digested with *Eco*RI

*Corresponding author. Fax: (49)-(0211)-8113706.

Abbreviations: ACMA, 9-amino-3-chloro-7-methoxyacridine; DCCD, *N,N'*-dicyclohexylcarbodiimide; F_0,F_1, intrinsic and extrinsic subcomplexes of the H^+-translocating ATPase; TTFB, 4,5,6,7-tetrachloro-2-trifluoromethylbenzimidazole.

SSDI S0014-5793(96)00167-6

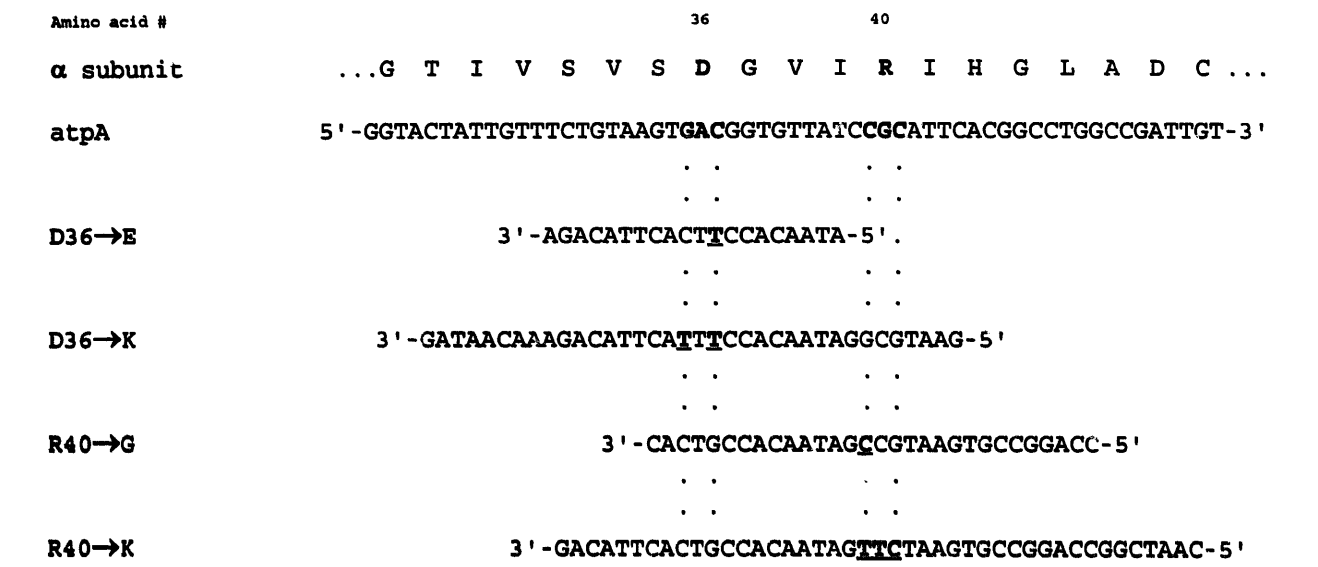

Fig. 1. Synthetic oligonucleotides for site-directed mutagenesis.The upper two lines show the mutated region of α subunit and of the *atp*A gene, respectively, of *E.coli* [3]. The following lines show the sequences of the employed synthetic oligonucleotides. The target codons in *atp*A and the respective amino acids are in bold letters; mutant bases in the oligonucleotides are shown in bold underlined letters.

and *Sma*I. The resulting 850 bp fragment was inserted into pBSCIIKS (Stratagene, La Jolla) which had been restricted with *Hin*dIII, treated with Klenow fragment to form blunt ends and subsequently cleaved with *Eco*RI. The resulting plasmid pBH21 was used for oligonucleotide-directed mutagenesis with the Double Take Doublestranded Mutagenesis Kit (Stratagene, La Jolla). Fig. 1 shows the mutated region of *atpA* and the corresponding amino acid sequence of α subunit as well as the employed synthetic oligonucleotides.

The mutated derivatives of pBH21 were verified by restriction analysis and sequencing according to the Pharmacia T7 DNA sequencing protocol. After cleavage with *Eco*RI and *Sfi*I the resulting 300 bp fragment comprising the mutated base triplets was subcloned into plasmid pBH2 to provide two restriction sites which could be used for cloning of the mutated fragments into pDP34. For construction of pBH2 an 857 bp fragment was isolated from pDP34 by digestion with *Sph*I and *Sma*I and cloned into pUC19 [7], which had been restricted with *Eco*RI, treated with Klenow fragment and finally cleaved with *Sph*I.

Fragments containing the mutated base triplets were subsequently isolated from pBH2 after restriction with *Sph*I and *Sfi*I. The resulting 308 bp fragments were cloned into pDP34 which had been restricted with *Sph*I and *Sfi*I. The resulting plasmids pDP34αD36→E, pDP34αD36→K, pDP34αR40→G and pDP34αR40→K were verified by sequencing and then used for complementation of the *atpA*-deficient *E. coli* strain RH304. The transformation was carried out with the medium of Chung et al. [8]. As a positive control, wild type cells were created by complementation of RH304 with pDP34, as a negative control RH304/pUC19 was used.

2.2. Membrane vesicle preparation

E. coli membrane vesicles were prepared by a modification of the method of Steffens et al. [9]. For every preparation freshly transformed cells were used. The cells were grown in 2YT medium containing 1.6% peptone, 1% yeast extract, 0.5% NaCl and 50 μg/ml ampicillin to a cell density of OD_{578nm} = 0.8. The cells were harvested by centrifugation at 10000×g for 10 min and resuspended in medium I containing 50 mM Tris-HCl pH 8.0 and 10 mM $MgCl_2$, to yield a density of 0.5 g wet cells/ml. Cells were disrupted by sonification with a Branson sonifier twice for 4 min 50% pulsed mode at 0°C. Whole cells and cell debris were removed by centrifugation at 27000×*g* for 20 min. The supernatant was then centrifuged at 300000×*g* for 1 h at 4°C. Finally, the pellets were resuspended in buffer I to an extent of 200 μl/g of initial wet cell weight.

2.3. Analytical tec iques

Protein concentrations were determined following the method of Dulley and Grieve [10]. For measurement of ATPase activity a discontinuous or the continuous assay of Arnold et al. [11] with on-line colorimetric determination of phosphate was employed. The medium contained 50 mM Tris buffer, pH 8.0, 1 mM $MgCl_2$, 0.6 μM nigericin and 1 mM ATP. ATP formation was measured discontinuously according to [12]. The medium contained 50 mM MOPS buffer, pH 7.0, 10 mM $MgCl_2$, 5 mM phosphate, 0.1 mM ADP. The reaction was started by the addition of 50 μM NADH. ATP was assayed by the luciferin/luciferase bioluminescence test as in [12]. The formation of an ATP- or NADH-induced proton gradient was monitored by the fluorescence quenching of ACMA in an Aminco Spectrofluorimeter

Table 1
Rates of ATP formation and ATP hydrolysis

Mutant	ATP formation			ATP hydrolysis			
	μmol/mg protein per min		% of wild type	μmol/mg protein per min		% of wild type	ATP formation / ATP hydrolysis
	Mean[a]	(S.D.)[b]		Mean	(S.D.)		
Wild type	0.153	(0.046)	100	0.518	(0.098)	100	0.30
HS1 (D36→E)	0.136	(0.049)	89	0.482	(0.148)	93	0.28
HS2 (D36→K)	0.035	(0.017)	23	0.144	(0.025)	28	0.24
HS3 (R40→G)	0.126	(0.039)	82	0.474	(0.127)	92	0.27
HS4 (R40→K)	0.130	(0.016)	85	0.489	(0.096)	94	0.27

[a]Mean values of 3–5 experiments.
[b]Standard deviation.

Table 2
Extents of the proton gradient generated by NADH oxidation or ATP hydrolysis, respectively

Mutant	NADH oxidation						ATP hydrolysis		
	% ACMA quench		% of wild type	+DCCD			% ACMA quench		% of wild type
	Mean[a]	(S.D.)[b]		% ACMA quench		% of wild type	Mean	(S.D.)	
				Mean	(S.D.)				
Wild type	84.5	(2.6)	100	78.0	(7.0)	100	78.0	(7.9)	100
HS1 (D36→E)	76.3	(5.3)	90	82.5	(3.5)	106	62.5	(10.5)	80
HS2 (D36→K)	56.0	(15.0)	66	78.5	(3.5)	101	14.0	(7.5)	18
HS3 (R40→G)	75.0	(9.2)	89	81.5	(1.5)	104	65.0	(4.9)	83
HS4 (R40→K)	79.0	(4.5)	94	79.0	(1.0)	101	76.0	(5.1)	97

[a]Mean values of 3–5 experiments.
[b]Standard deviation.

SPF 500 or in a self-constructed fluorimeter following the method of Schairer et al. [13]. The excitation wavelength was 410 nm, fluorescence emission was measured at 490 nm. The assay medium contained 20 mM Tricine/NaOH pH 8.0, 10 mM $MgCl_2$ and 300 mM KCl. The vesicle protein concentration was 0.4 mg (ATP-induced) or 0.2 mg (NADH-induced) in a total volume of 2 ml. The temperature was 37°C. After the addition of 1 μM ACMA the reaction was started with 1 mM ATP or 0.1 mM NADH, respectively. The fluorescence quench due to physical interaction of nucleotides with ACMA was ascertained by the signal height obtained upon addition of 2 μM of the uncouplers TTFB or nigericin.

For quantification of the content of F_1, vesicle suspensions were subjected to SDS-PAGE [14] using a 12% gel. After treatment with Coomassie Brilliant Blue, the extracted stain of the excised band of subunit β was colorimetrically determined as described by Fiedler et al. [15]. This subunit was chosen because it was not superimposed by other protein bands.

Immunoblotting with anti-$EF_1\alpha$ following SDS-PAGE was performed with a Nova-Blot (LKB) semi-dry blotting system as described by the supplier. Detection by the Enhanced Chemiluminescence System (ECL) was carried out according to the protocol of Amersham.

3. Results

The acid amino acid α-D36 was replaced either by glutamate having similar physicochemical character (α-D36→E in transformant HS1), or by the oppositely charged lysine (α-D36→K in transformant HS2). The basic amino acid α-R40 was changed to either lysine (α-R40→K in transformant HS4) or glycine (α-R40→G in transformant HS3). The proteins of the transformed cells were subjected to SDS-PAGE. Immunoblotting with an antiserum against α subunit of F_1 from *E. coli* indicated that α subunits were expressed in virtually equal amounts in all complemented cells, whereas the F_0F_1-deficient acceptor strain RH 304 was completely free of α.

Table 3
Relative F_1 contents of wild type and HS2 vesicle membranes

	A_{595nm} of excised β bands	
	Unwashed vesicles	Washed vesicles
Wild type	0.124	0.113
HS2 (α-D36→K)	0.124	0.108

Washing of the vesicles was carried out with isolation buffer (medium I, cf. section 2). The vesicles were subsequently dissolved in SDS buffer and subjected to SDS-PAGE. Extraction and colorimetric measurement of Coomassie dye from the excised β-bands were conducted as described [15].

No significant differences in growth rates were observed between the wild type and the transformants when grown fermentatively on glucose. For investigation of the mutated F_0F_1 complexes, plasma membrane vesicles were prepared. Table 1 shows rates of ATP formation of the vesicles coupled to NADH oxidation and rates of ATP hydrolysis. Both reactions show parallel sensitivity against mutation, but the effect on ATP formation was slightly higher. In the mutant HS2 (α-D36→K) phosphorylation and ATP hydrolysis are 77% and 72%, respectively, inhibited whereas in the other transformants phosphorylation is less than 20% inhibited and ATP hydrolyzing activity was less than 10% inhibited. The ratio of ATP formation over ATP hydrolysis may be taken as a measure of coupling. This ratio is significantly lower in the mutant HS2, but less strikingly different in the other mutants.

More precise information on coupling may be obtained by measurement of proton gradient formation. The generation of a proton gradient at the expense of NADH oxidation was monitored by ACMA fluorescence quenching. The extents of the proton gradients were similar in three of the mutants and accounted for at least 89% of the wild type, but in HS2 the gradient was substantially lower (Table 2). After treatment with DCCD to block proton leakage through F_0, however, all mutants attained the same extent of the gradient as the wild type. We have measured the respiratory activity of the vesicles and found no differences in the rates of uncoupled NADH oxidation suggesting identical proton pumping activity of all transformants and the wild type. In summary, the results indicate differences in proton leakiness of the F_0F_1 complexes. Leakiness is particularly striking in the mutant HS2.

Table 2 furthermore shows the extents of the proton gradients formed at the expense of ATP hydrolysis. This activity should be additively affected by inhibition of the catalytic process and proton leakiness, and thus be rather sensitive to mutation. Comparison of the mutants shows that the exchanges of α-R40 are least inhibitory, the exchange of α-D36→K, however, inhibits the ATP-induced proton gradient formation by 82%.

The reduced ATPase activity of mutant HS2 might be due to a reduced F_1 content of the vesicle membranes although the Western blot analysis of α subunits did not indicate a loss

of F_1 (see above). To confirm this result, the protein patterns of wild type and mutant membranes were quantitatively compared. As in SDS-PAGE of membrane vesicles subunits β is not superimposed by other protein bands, this band is suitable for quantitative analysis by colorimetry of the extracted stain. The results (Table 3) show the same staining intensity in wild type and mutant HS2. However, it seems that the mutant F_1 is slightly more sensitive to washing with isolation medium. Washing with 0.5 mM EDTA in a buffer of low ionic strength which causes rather specific release of F_1 resulted in 80% release of ATPase activity into the supernatant. The activities were 1.27 and 0.48 μmol P_i/mg protein per min for the wild type and for HS2, respectively, suggesting that the specific ATPase activity of the mutated F_1 is indeed reduced. It seems, however, that the activity of the released F_1 is less affected by this mutation (62% inhibition) than the ATPase activity of the membrane-bound F_1 (72% inhibition).

4. Discussion

The crown of F_1 is formed by six-stranded β-barrels of the alternately arranged α (*E. coli*: amino acids 19–95) and β subunits (amino acids 1–75). The six strands are designated a–f [4]. Each a-strand is hydrogen-bonded to the d-strand of a neighbouring subunit, forming a continuous β-sheet. The aspartic acid α-D36 is located in the a-strand of α subunit where it adjoins the d-strand of a β subunit. A conserved basic amino acid, the arginine β-R52 is located in the d-strand of β subunit. Both charged residues, that of α-D36 and that of β-R52, may protrude into the interior of the $\alpha_3\beta_3$ ring and may be close to each other. A specific interaction of this domain of the crown with the carboxy-terminal helix of γ subunit is not likely because the last two α-helical turns of γ which might be close enough to α-D36 consist of uncharged, mostly hydrophobic amino acids. On the other hand, the replacement of α-D36 by the basic amino acid lysine may cause destabilization of the $\alpha_3\beta_3$ ring by an electrostatic repulsive effect with β-R52. It should also be mentioned that we found that coupling of the vesicles of mutant HS2 is more sensitive to freeze-thawing which might be referred to such a disturbance of subunit interaction. The repulsion, however, is not strong enough to prevent the assembly of the complex.

Obviously, the F_0F_1 complex becomes more leaky for H^+ when α-D36 is replaced by K. Since the leakiness is prevented by the F_0 blocker DCCD, it is probable that this mutation changes the interaction between F_0 and F_1 so that about one third of the protons flow out through F_0 in a non-productive manner (cf. Table 2). This is surprising since the crown is located opposite to the F_0 binding pole [4] and indicates that coupling requires a precise fine structure of the entire F_0F_1 polypeptide complex. The structural significance of the N-terminal end of α subunit is supported by earlier reports indicating that proteolytic removal of amino acids 1–30 [16] or mutation of the conserved amino acid α-G29 [6] caused disturbance of F_1 binding to F_0.

Besides coupling catalysis is restricted to the same or even higher extent in HS2 (cf. Table 1). As the crown is about 50 Å away from the catalytic sites, the effect is of course indirect, too. The catalytic sites are located on the β subunits, but at the interface to the α subunits and the adjacent domains of the α subunits may participate in catalysis [4]. It is quite plausible that stretching of the $\alpha_3\beta_3$ ring due to electrostatic repulsion would affect the conformation of the catalytic sites. Hence, the effect of α-D36→K replacement on two different functions can be traced back to the same structural cause.

Acknowledgements: This work was supported by Deutsche Forschungsgemeinschaft (Sonderforschungsbereiche 189 and 171) and Fonds der Chemischen Industrie. The authors are grateful for providing mutant strains, plasmids and antibodies to Prof. A. Senior and Prof. K. Altendorf. They thank F. Bracht for construction of oligonucleotide α-D36→E.

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