ISSN 0006-2979, Biochemistry (Moscow), 2006, Vol. 71, No. 9, pp. 1006-1012. © Pleiades Publishing, Inc., 2006.
Original Russian Text © S. V. Ponomarenko, 2006, published in Biokhimiya, 2006, Vol. 71, No. 9, pp. 1234-1242.

Biochemical Characteristics of *Escherichia coli* ATP Synthase with Insulin Peptide A Fused to the Globular Part of the γ-Subunit

S. V. Ponomarenko

Institute of Plant Biochemistry of Heinrich Heine University, Dusseldorf, Universitatsstr. 1, 40225 Dusseldorf, Germany; fax: (49) 211-8113706; E-mail: ponomarenko@t-online.de

Received March 15, 2006
Revision received May 8, 2006

Abstract—The codon 5383-5385 (CCG) in the *atpC* gene of the *unc* operon of *Escherichia coli* cells was replaced with the sequence encoding peptide A of human insulin. The foreign protein fused to the middle part of the γ-subunit of ATP synthase affects neither biosynthesis of the chimeric polypeptide nor the integration of the $EF_0{\cdot}F_1$ enzyme into the membranes of the *E. coli* cells. The inserted peptide A does not inhibit the process of oxidative phosphorylation. The ATPase activity of the mutant $EF_0{\cdot}F_1$ enzyme was equal to that of the wild-type enzyme and was regulated by modifiers in the similar way, suggesting that the space in the stalk area of F_0/F_1 interaction is enough for the introduction of an additional oligopeptide without changing catalytic properties of the ATP synthase.

DOI: 10.1134/S0006297906090094

Key words: γ-subunit of ATP synthase, insulin peptide A, chimeric protein expression

ATP synthase ($F_0{\cdot}F_1$) is an enzyme integrated into the energy-coupling membranes of cells and catalyzes synthesis (hydrolysis) of ATP. ATP synthase from bacteria ($BF_0{\cdot}F_1$), mitochondria ($MF_0{\cdot}F_1$), and chloroplasts ($CF_0{\cdot}F_1$) includes the membrane-integrated subcomplex F_0 and the catalytic subcomplex F_1 connected with F_0. Both parts of the enzyme molecule have oligomeric structure. The subcomplex F_1 is usually composed of nine polypeptides of the following stoichiometry: $\alpha_3\beta_3\gamma\delta\varepsilon$ [1-5]. The molecular mechanism of the reaction of ATP hydrolysis is similar for bacterial, mitochondrial, and chloroplast ATP synthases, this being confirmed experimentally. Molecular-biological and biophysical methods have demonstrated that the catalytic hydrolysis of ATP is accompanied by the rotation of the γ-subunit in hexameric F_1, which provides incremental movement of the rotor part $\gamma\varepsilon c_{12}$ inside the stator part $\alpha_3\beta_3\delta ab_2$ [1, 5-7]. Further confirmation of the mechanism of the enzymatic catalysis was connected with the detection of the redox-dependent rotation of the chimeric γ-subunit, which accompanied ATP hydrolysis by a single molecule of ATP synthase isolated from the thermophilic bacterium *Bacillus* PS3 [8]. The influence of the formation of the disulfide bond γCys199–Cys205 in the γ-subunit of $CF_0{\cdot}F_1$ from spinach on the rate of its rotation in the bacterial catalytic hexamer $\alpha_3\beta_3$ was investigated.

The γ-subunit can be divided relatively into three structurally and functionally different parts. In the γ-subunit of *E. coli*, these are two α-helical N- and C-terminal fragments of 80 and 120 Å, respectively, and also the medial globular fragment composed of 150 amino acid residues [9-11]. Both α-helixes are extended from the membrane subcomplex F_0 within the space formed by the catalytic hexamer $\alpha_3\beta_3$, the globular part of the γ-subunit being located between F_1 and F_0 [9, 11].

The γ-subunit of the mitochondrial and chloroplast ATP synthases is encoded by the nuclear genome, which determined evolutionary changes in its gene [3, 4, 12]. The medial fragment of the γ-subunit of ATP synthase from the photosynthetic cells is 30 amino acid residues longer compared to that of $BF_0{\cdot}F_1$ and $MF_0{\cdot}F_1$ [4, 13, 14]. The additional fragment of the γ-subunit is similar in ATP synthases of chloroplasts and blue-green algae. It was named "photosynthetic motif" for its participation in light regulation of the activity of ATP synthases.

Abbreviations: *atpC*) gene encoding γ-subunit of ATP synthase; $atpC^{inAH}$) mutant gene with inserted DNA of insulin peptide A and Histag; $EF_0{\cdot}F_1$) ATP synthase of *E. coli* cells; EF_1 and EF_0) catalytic and membrane-integrated subcomplexes of ATP synthase, respectively; Histag) several histidine residues on the terminus of a polypeptide; *unc*) operon encoding all subunits of $EF_0{\cdot}F_1$; LB) Luria–Bertani medium; wt) wild type; γEwtH) clone of *E. coli* cells containing ATP synthase with the γ-subunit with Histag on its C-terminus.

The ATP synthases $MF_0{\cdot}F_1$, $BF_0{\cdot}F_1$, and $CF_0{\cdot}F_1$ from different species are investigated in several laboratories using different methods including gene engineering. The γ-subunit of ATP synthase is a convenient subject for structural and functional analysis. Recently, it has been shown that the deletion of 17 amino acid residues of the photosynthetic motif (γ210D-226R) affects two important characteristics of chloroplast ATP synthase, suppressing its catalytic activity and decreasing its ability for generating the transmembrane proton gradient (ΔpH) [15]. Besides, it was demonstrated that the rate of ATP hydrolysis by the chimeric bacterial TF1 $(\alpha_3\beta_3)_T$-γ_C complex reconstituted from the catalytic hexamer $\alpha_3\beta_3$ of ATP synthase of the thermophilic bacterium PS3 and the chloroplast γ-subunit without the photosynthetic motif γP194-I230 (spinach) was almost twofold lower [16].

Another chimeric ATPase designated as $[\alpha_3\beta_3]_T\gamma_{TCT}$, where the fragment Val192-Phe202 of the γ-subunit of the thermophilic bacterium PS3 was replaced with a homologous fragment of 148 amino acid residues (Val196-Phe243) from spinach $CF_0{\cdot}F_1$, was physiologically active [13, 17].

It is not known whether an oligopeptide analogous to the photosynthetic motif fused to the γ-subunit is able to alter the properties of $EF_0{\cdot}F_1$.

The goal of the present study was to investigate the effect of human insulin peptide A fused to the globular part of the γ-subunit of ATP synthase from *E. coli* on catalytic and regulatory properties of this enzyme.

MATERIALS AND METHODS

In the present work the cloning method described by Maniatis [18] was used. The primary structure of all inserted DNA sequences obtained by the PCR method was checked by their sequencing. Bacterial strains XL-Blue (Stratagene, USA), DK8 [19], and EZ (Qiagen, Germany) and plasmid vectors pDrive (Qiagen), pBR322 [20], pBBWU13 [21], peHγ, and pUγHwt were used as described in the previous work [22].

Amplification of DNA was performed according to the HotStar PCR method (Qiagen) [23]. PCR was performed in 25 or 50 μl of the HotStar buffer solution containing Qiagen HotStar DNA polymerase, 5-10 ng DNA, 2 mM $MgCl_2$, 250 nmol of each primers, and 250 μM dNTPs. The primers were synthesized and purified using HPLC by BWG (Germany). The resulting PCR product was purified electrophoretically with subsequent extraction from the agarose gel using a Mini Elute Kit (Qiagen) [23].

The recombinant DNA was cloned using Cloning Kit (Qiagen) [23]. For this purpose, the highly purified DNA fragments were inserted into the pDrive vector, and the resulting plasmids were transformed into competent *E. coli* cells (strain EZ). The eluted and purified DNA was inserted using Quick Ligation Kit [23] (15-30 min at 5-8°C). The competent cells were transformed using the heat shock method [18]. The highly purified plasmid DNA was obtained by anion-exchange chromatography using a QIAprep Mini Prep Kit [23].

Resistance to antibiotics ampicillin and kanamycin was used for the selection of the mutant clones. Tanaka minimal medium [24] was used to investigate the ability of *E. coli* cells to grow under the conditions of oxidative phosphorylation. For this purpose, the corresponding plasmids were transformed into the DK8 competent cells lacking the *unc* operon [19]. The transforming mixture was centrifuged for 2 min at 1500*g*, and then the treated cells were resuspended gently in 10 mM Tris-HCl buffer, pH 8.0, and plated onto Petri dishes with Tanaka minimal medium containing 0.4% succinate as the only source of carbon. The cells were incubated on Petri dishes or in liquid culture for 24-72 h at 37°C.

Cell membranes were isolated and purified according to Schnick et al. [25]. EF_1 ATPase was extracted from the cell membranes with chloroform as described by Younis [26] with subsequent purification on Ni-NTA Spin Columns [23].

To obtain $EF_0{\cdot}F_1$, the membrane particles after ultracentrifugation were resuspended in 4 ml of medium for isolation (20 mM TES-NaOH, pH 7.0, 5 mM $MgCl_2$, 150 mM KCl, 0.5% (w/v) ε-aminocaproic acid, 10% (v/v) glycerol, 0.002% phenylmethylsulfonyl fluoride). The ATP synthase complex was solubilized in the indicated medium containing octyl-β-D-glucopyranoside (final concentration, 2%) under the following conditions: 2 min at 37°C, 4 min on a shaker (200 U/min), 1 min at 37°C, 1 min on the shaker, and then 30 min at 4°C on a magnetic stirrer (100 rpm). The solubilized proteins were separated from the membrane fraction by ultracentrifugation for 1 h at 278,000*g* and 4°C. The supernatant was used for the purification of the enzyme by immobilized-metal affinity chromatography Ni-NTA. The eluting buffer contained the medium for isolation, 0.02% octyl-β-D-glucopyranoside and 10-200 mM imidazole.

To remove components that could affect the determination of the ATPase activity, the enzyme was purified by gel filtration on a Bio-Spin column (Bio-Rad, USA) equilibrated with 20 mM Tricine, pH 8.0, containing 150 mM NaCl. The ATPase activity was determined by the accumulation of inorganic phosphate at 37°C as described by Schnick et al. [25]. The reaction mixture (1 ml) contained 20 mM Tricine, pH 8.0, 150 mM KCl, 50 μg of protein, 5 mM $MgCl_2$, and 2 mM ATP. The reaction was started by addition of the substrate (Mg-ATP).

Proteins were analyzed by SDS-PAGE with subsequent staining with Coomassie R-250, or were transferred onto a PVDF membrane (Schleicher & Schuell, Germany) for immunoblotting using specific rabbit monoclonal antibodies against the γ-subunit of the subcomplex EF_1. The antibodies were detected with BM

Bioluminescent Blotting Substrate (POD) (Roche, Switzerland). To determine molecular weights of the polypeptides transferred onto the membrane, Bio-Rad Prestained Standards (250-10 kD) were used.

Protein content in the range of 2-10 μg/ml was determined using the Bio-Rad Protein Assay method using BSA as the standard.

The following computer programs were used: ClustalX, for alignment procedure [27]; Chromas2, for checking sequences; Winclone, for construction of oligomeric primers and finding restriction sites of DNA; PATPS, for correspondence of codons [28]; P2Gen, for finding homologous proteins.

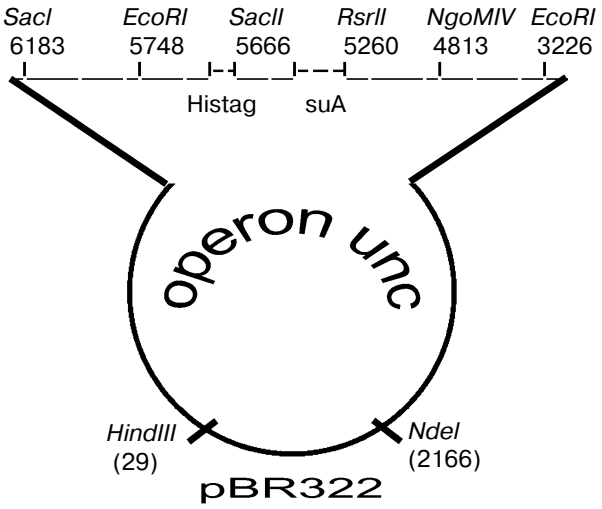
RESULTS AND DISCUSSION

Expression vector pgEinAH. The inserted recombinant polypeptide was chosen among known proteins using the program P2Gen so that its length, amino acid composition, and tertiary structure would be analogous to that of the chloroplast photosynthetic motif. Peptide A of human insulin fits these requirements [12, 29].

It should be noted that physiologically active insulin is composed of two subunits, A and B (21 and 30 amino acid residues, respectively). This human proteohormone was the first protein obtained by a pharmaceutical firm using recombinant DNA. Several industrial methods of expression and purification of the recombinant insulin were developed that became model approaches for production of many other proteins using gene engineering [29].

To obtain and to clone the expression vector, the following methods were used: PCR, restriction analysis, genetic insertion, and transformation.

To synthesize the recombinant protein, the plasmid pgEinAH vector (see Scheme) was constructed by site-directed mutagenesis using the pBWU13 plasmid obtained by Futai [21] as the template DNA. The plasmid pBWU13 DNA contains the *unc* operon for biosynthesis of all subunits of the wild-type *E. coli* ATP synthase lack-



Scheme of the transforming vector pgEiAH. The pgEiAH vector contains the mutant $atpC^{inAH}$ gene in the area of the restriction sites *Ngo*MIV-*Eco*RI of the *unc* operon [2]. The scheme shows the position of Histag, the gene encoding the subunit A of human insulin (suA), and the restriction sites discussed in the text

ing the *Nde*I restriction site. To insert the DNA sequence corresponding to the peptide A of human insulin, two DNA fragments encoding C- and N-flanks of the γ-subunit were amplified. PCR was performed according to the Qiagen HotStar PCR procedure [23] using the primers presented in Table 1. The oligomeric primers were constructed so that the amino acid residues of the human protein (Fig. 1) would be encoded by the codons used in the bacterial cell. For this purpose, the program PATPS was used [28].

Figure 2 shows the PCR products for the first (fA) and the second (eA) parts of the DNA sequence encoding peptide A within the γ-subunit (Scheme). Each of the PCR products fA and eA was inserted into the pDrive vector (Qiagen), and the resulting plasmids were named pfA and peA, respectively. To construct the pAfe vector, the restriction fragment *Nde*I-*Xho*I of the pfA plasmid

Table 1. Primers for the site-directed mutagenesis of the *atpC* gene of *E. coli* cells

Primer	DNA sequence
Eg1for	5'-ACCGCGGATG***GCCGGC***GCAAAAGAGATACG
NdeArev	5'-CCGCGG***CATATG***CTGGTGCAGCACTGTTCAACAATGCCCATGG GTAACGGCAGCAGCTGGCTG
forANde	5'-G***CATATG***CAGCTTGTATCAGTTGGAAAATTATTGCAATAGGCGC GC*A*TCAGATGATGATGATCTG
SacIrev	5'-CGAAT***GAGCTC***CATCATGTTTAC

Note: The restriction sites *Nde*I (***CATATG***), *Ngo*MI (***GCCGGC***), and *Sac*I (***GAGCTC***) are shown bold.

```
5383                                                    5386
CccAtgGgcAttGttGaaCagTgcTgcAccAgcAtaTgcAgcTtgTatCagTtgGaaAatTatTgcAatAggCgcGcaTcaGatGatGat
  P  M  G  I  V  E  Q  C  C  T  S  I  C  S  L  Y  Q  L  E  N  Y  C  N  R  R  A  S  D  D  D
192                                                     193
```

Fig. 1. Amino acid sequence of the heterogeneous oligopeptide fused to the γ-subunit of *E. coli* ATP synthase. The amino acid sequence of peptide A of human insulin (G1-N21) inserted between P192 and A193 of the γ-subunit of *E. coli* ATP synthase is underlined. The DNA fragment inserted between cytosine 5383 and guanine 5386 of the *unc* operon is shown bold; *cAtaTg* is the restriction site of *Nde*I.

(0.6 kb) was inserted into the prepared fragment of the peA plasmid with the corresponding sticky ends *Nde*I and *Xho*I. The pAfe plasmid contains the DNA sequence encoding the hybrid γ-subunit of *E. coli* ATP synthase. The plasmid pAfe transformed into XL-Blue cells contains the *Nde*I restriction site in the area of the inserted fragment of the foreign DNA (Fig. 1).

The restriction fragment *Rsr*II + *Sac*II (Scheme) of the pAfe plasmid (0.5 kb) was inserted into the previously created peHγ vector [22] containing the unique restriction sites *Rsr*II and *Sac*I in the DNA of the *unc* operon. The DNA of the peHγ vector differs from the wild-type DNA in 15 nucleotides encoding histidine residues for the formation of a Histag on the C-terminus of the γ-subunit. The resulting pASR2 plasmid is a product of pDrive vector with the integrated section *Rsr*II + *Sac*I of the *unc* operon. To obtain the plasmid vector containing the full *unc* operon, the indicated fragment *Rsr*II + *Sac*I was inserted into the pBWU13 vector pretreated with the corresponding endonucleases, and the resulting plasmid was named pgEiAH (Scheme). To obtain the mutant γEinAH strain, the competent DK8 *E. coli* cells deficient in the *unc* operon (1100Δ[uncB-uncC]ilv::Tn10) [19] were transformed with the pgEiAH vector. For the control, two plasmids pBR322 and pBWU13 were transformed in the DK8 cells, resulting in the corresponding clones: zero *unc* operon (DK8*) and wild type (wt*).

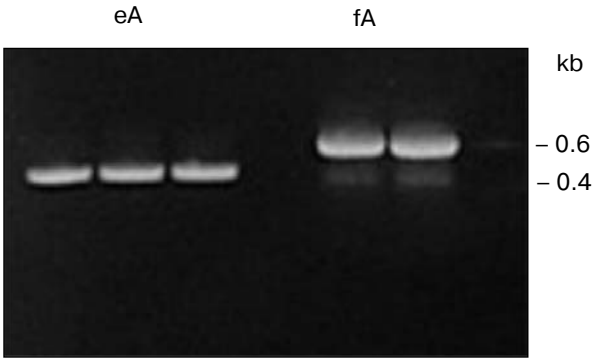


Fig. 2. Electrophoresis of the amplified DNA fragments. The eA and fA DNA fragments obtained by the PCR method (see "Materials and Methods") and used to create the pAfe vector were separated by electrophoresis in 1% agarose. The size of the standards is indicated in kb on the right.

Phenotypic properties of the mutant cells γEinAH and biochemical characteristics of their ATP synthase. The DNAs of the pgEinAH and pUγHwt plasmids [22] must differ from that of pBWU13 in the length of their 3226*Eco*RI-5748*Eco*RI restriction fragments of the *unc* operon due to the elongation by 15 nucleotides in pUγHwt [22] and by 87 nucleotides in pgEinAH. Restriction analysis confirms the elongation of the indicated DNA fragment in the pgEinAH vector (Fig. 3). Besides, pgEinAH has the additional restriction site *Nde*I (Fig. 1) that is absent in the pBWU13 [21].

To confirm the expression of the mutant *E. coli* ATP synthase, its catalytic subcomplex EF_1 was isolated using

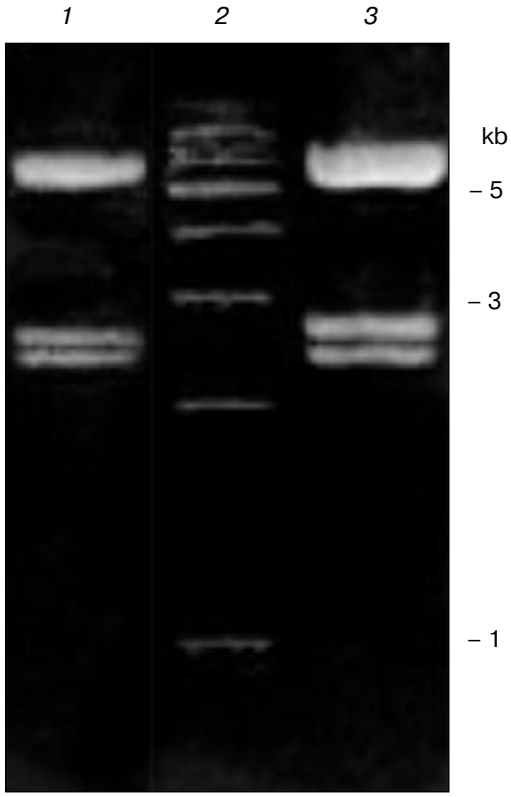


Fig. 3. Restriction analysis of the gEwtH and gEinAH vectors. The gEwtH and gEinAH plasmids were restricted using *Eco*RI endonuclease. Electrophoresis was performed in 1% agarose: *1*) gEwtH; *2*) DNA standard; *3*) gEinAH. The size of the standards is indicated in kb.

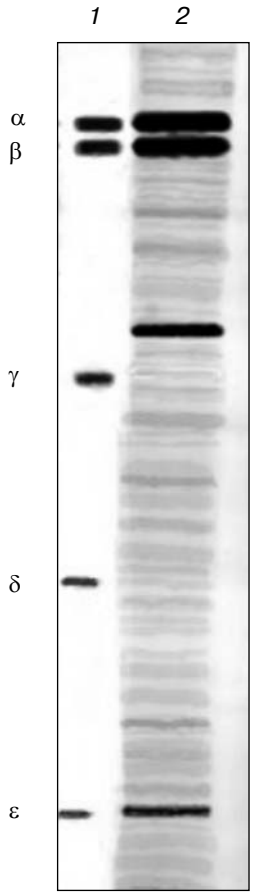


Fig. 4. Electrophoretic mobility of the EF_1 subunits. SDS-PAGE was performed in 4-20% polyacrylamide gel. ATPase was purified from the *E. coli* cells: *1*) wild-type enzyme (standard) isolated by the Schnick method [25] (4 μg); *2*) the enzyme isolated from the γEinAH cells after the extraction with chloroform and subsequent purification on a Ni-NTA Mini Spin column (25 μg) (see "Materials and Methods"). The protein bands were stained with Coomassie R-250. The subunits of EF_1 are indicated on the left.

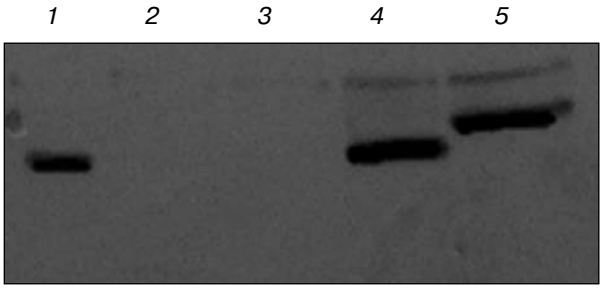


Fig. 5. Immunoblotting of the γ-subunit of *E. coli* ATP synthase. The antibodies against the γ-subunit of *E. coli* ATP synthase were used for the staining. SDS-PAGE was performed in 15% polyacrylamide gel. Membrane proteins of the following cell lines were applied onto the gel: *1*) wild type (3 μg); *2*) DK8; *3*) DK8*; *4*) γEwtH; *5*) γEinAH (in *2-5*, 10 μg of protein was applied).

the Ni-NTA Mini Spin method (Qiagen) developed for quick isolation of a protein containing Histag. The elution yielded a protein solution enriched with EF_1. Figure 4 illustrates the difference in the molecular weight between the γ-subunits of ATP synthase from the wild-type and γEinAH cells. The polypeptide encoded by the mutant gene $atpC^{inAH}$ moves slower, so it contains the inserted recombinant protein, this supporting the data of the restriction analysis (Fig. 3) on the expression of the mutant *E. coli* ATP synthase. Western blotting demonstrated that the membranes of the wild-type as well as both mutant cells γEwtH and γEinAH contained almost the same amount of the γ-subunit (Fig. 5). In contrast, the cell lines DK8 and DK8* did not bind the antibodies against of the γ-subunit (Fig. 5).

The inserted recombinant protein does not affect the extraction of the catalytic subcomplex EF_1 with chloroform. The obtained EF_1 lacks the δ-subunit (Fig. 4), as the other soluble ATPases isolated using the indicated method [26].

The presence of the active ATP synthase in the energy-coupling cell membranes is usually confirmed by the ability of the cells to grow on a minimal medium using the process of oxidative phosphorylation. In the present work, Tanaka minimal medium [24] supplemented with 0.4% succinate as the only source of carbon was used. The γEinAH cells, analogous to those of the wild type and γEwtH [22], grow on minimal media (Table 2), suggesting that the mutant with the inserted recombinant protein can use succinate as the source of carbon and its ATP synthase catalyzes oxidative phosphorylation in contrast to the DK8* cells (zero *unc*). Immunoblotting with the use of the antibodies against the γ-subunit of EF_1 supports that the γ-subunits of ATP synthase of the clones γEinAH, γEwtH, and wt differ in their electrophoretic mobility in SDS-PAGE (Fig. 5). The chimeric polypeptide exhibits the lowest electrophoretic mobility, which corresponds to the highest molecular weight, this being in agreement with the data of the restriction analysis of DNA (Fig. 4).

The cells of the mutant γEinAH *E. coli* clone have a mutation in the *atpC* gene of the *unc* operon, where the triplet CCG (5383-5385) encoding a proline residue is replaced with the gene of peptide A of human insulin (63 bp) (Fig. 1). The recombinant peptide is inserted between Pro192 and Ala193 of the γ-subunit. The presented tertiary structure of the γ′ε dimer performed with high resolution [10] indicates that the inserted peptide must be located in the area of the medial globular fragment before the α_6-helix of the C-terminus threading the catalytic hexamer $\alpha_3\beta_3$ of the subcomplex F_1 [9, 11, 30]. It was shown that the contact between the parts F_0 and F_1 of the oligomeric ATP synthase complex is provided by their electrostatic interaction [31]. Amino acid residues of the globular fragment of the γ-subunit are involved in the interaction between the catalytic subcomplex F_1 and the membrane-integrated F_0 [11, 32, 33]. The inserted

Table 2. Growth rate of *E. coli* cells depending on the nutrient medium and type of *unc* operon

Type of *unc* operon	Strain	Growth on minimal medium	Time for two-fold increase in the cell biomass, %
No	DK8*	—	142
wt*	wt*	++++	100
γ + Histag	γEwtH	++++	104
γ + suA + Histag	γEinAH	++++	109

Note: The cells were grown on Tanaka minimal medium [23] containing 1.5% agar for 2-3 days at 37°C. The growth rate of the liquid culture was determined spectrophotometrically by the absorption at 600 nm. The cells were grown at 37°C in LB medium containing 0.2% glucose. The time for the two-fold increase in the cell biomass for wt* was 46 min and taken for 100%.

Table 3. ATPase activity of $EF_0 \cdot F_1$ from cells γEwtH and γEinAH

Clone	Without modifier	10 mM Na_2SO_3	5 mM NaSCN
γEwtH	0.329	0.599	0.060
γEinAH	0.328	0.598	0.058

Note: ATPase activity (μmol ATP/mg protein per min) was determined by the method of Schnick and coauthors [25]. The reaction mixture (1 ml) contained 20 mM Tricine, pH 8.0, 150 mM KCl, 50 μg of the protein, 5 mM $MgCl_2$, and 2 mM ATP. The reaction was started by the addition of Mg-ATP.

recombinant protein does not destroy this interaction. In contrast, the heterologous insertion in the *AtpC* gene encoding chloroplast γ-subunit blocks both the transcription of the gene and the assembly of ATP synthase in the thylakoid membranes [34]. At the same time, the chimeric ATPase from the thermophilic bacterium PS3 $[\alpha_3\beta_3]_T\gamma_{TCT}$ reconstituted from the $\alpha_3\beta_3\varepsilon$ subunits of TF_1 and its γ-subunit, where the globular fragment γVal192-Phe202 was replaced with the γLeu96-Phe243 fragment of the homologous protein from spinach chloroplasts, was completely active, although it differed in its properties from both the bacterial and the chloroplast enzymes [13]. In the subsequent work, the indicated mutant was used to demonstrate that the deletion of three negatively charged amino acid residues 210EDE212 in the chloroplast part of the chimeric γ-subunit of the ATPase complex decreased the rate of the ATPase reaction and decelerate the rotation of the γ-subunit in the presence of thiols. This effect is opposite to the action of dithiothreitol and thioredoxin on chloroplast ATPase [4, 17].

Although the cells of the mutant clone γEinAH grew in LB medium more slowly than the wild-type cells (Table 2), the genetically inserted recombinant human protein did not alter the expression of the hybrid $atpC^{inAH}$ gene, which was confirmed by immunoblotting (Fig. 5). The recombinant protein affected neither the incorporation of the ATP synthase into the membranes of *E. coli* cells (Fig. 4), nor the process of oxidative phosphorylation (Table 2). The enzymes $EF_0 \cdot F_1$ of the wild-type and mutant cells hydrolyzed ATP with almost the same rates (Table 3). In the presence of 10 mM sulfite, the activity of ATPases isolated from both *E. coli* strains increased approximately twofold, and 5 mM thiocyanate inhibited the activity by more than 80% (Table 3). The used modifiers similarly affected the ATPases from chloroplasts $CF_0 \cdot F_1$ and mitochondria $MF_0 \cdot F_1$ [35].

The analysis of the primary structure of the γ-subunit of EF_1 and CF_1 [14], the data on the X-ray analysis of its tertiary structure [9-11, 30], and the properties of the enzyme with the genetically modified γ-subunit [12-17], as well as the results obtained in the present work indicate that the space in the area of the contact between the membrane-integrated F_0 and catalytic F_1 subunits of the bacterial ATP synthase is sufficient to accept a polypeptide of more than 20 amino acid residues. This space could be used in the process of adaptive evolution for the elongation of the medial part of the γ-subunit of the chloroplast enzyme. The recombinant human peptide is expressed as the part of the hybrid polypeptide and can be purified together with *E. coli* ATP synthase complex using the methods developed for isolation of F_1. To simplify the isolation of the recombinant protein, methionine and arginine residues were added (Fig. 1).

Investigation of the biochemical characteristics of ATP synthase of γEinAH cells demonstrated that the alien protein analogous to the chloroplast photosynthetic

motif increased the medial part of the γ-subunit of EF_1 by 24 amino acid residues. This neither blocked the expression of the chimeric γ-subunit of ATP synthase, nor altered the catalytic activity of the enzyme. The recombinant peptide A of human insulin inserted in the area of the EF_0/EF_1 contact does not change the catalytic activity of the enzyme, in contrast to the integrated chloroplast fragment with the γ210EDE212 deletion [17]. Consequently, such an insertion must not affect the rotation rate of the chimeric γ-subunit in the hexamer $\alpha_3\beta_3$ of ATP synthase during the catalytic hydrolysis of ATP.

The author is grateful to the Director of the Institute of Plant Biochemistry of Heinrich Heine University (Dusseldorf), Prof. H. Strotmann, for his constant support and for the possibility of working in the Institute, to Prof. G. Groth (from the mentioned institute) for the pBWU13 plasmid vector and DK8 *E. coli* cells, to Dr. M. Rost for the antibodies against the γ-subunit of *E. coli* ATPase and for the standard EF_1, and to I. N. Ponomarenko (Vesser) for the computer program P2Gen.

This work was performed in the frame of the project DFG Str 103/21-1 and supported by the Fund for Chemical Industry (Germany).

REFERENCES

1. Weber, J., and Senior, A. E. (2003) *FEBS Lett.*, **545**, 61-70.
2. Walker, J. E., Saraste, M., and Gay, N. J. (1984) *Biochim. Biophys. Acta*, **768**, 164-200.
3. Skulachev, V. P. (1989) *Membrane Bioenergetics*, Plenum Press.
4. Strotmann, H., Shavit, N., and Leu, S. (1998) in *The Molecular Biology of Chloroplasts and Mitochondria in Chlamydomonas*, Kluwer Academic Publishers, pp. 477-500.
5. Yoshida, M., Muneyuki, E., and Hisabori, T. (2001) *Nature Rev.*, **2**, 669-677.
6. Noji, H., Yasuda, R., Yoshida, M., and Kinosita, K., Jr. (1997) *Nature*, **386**, 299-302.
7. Sabbert, D., Engelbrecht, S., and Junge, W. (1996) *Nature*, **381**, 623-625.
8. Bald, D., Noji, H., Yoshida, M., Hirono-Hara, Y., and Hisabori, T. (2001) *J. Biol. Chem.*, **276**, 39505-39507.
9. Abrahams, J. P., Leslie, A. G. W., Lutter, R., and Walker, J. E. (1994) *Nature*, **370**, 621-628.
10. Rodgers, A. J. W., and Wilce, C. J. (2000) *Nature Struct. Biol.*, **7**, 1051-1054.
11. Gibbons, C., Montgomery, M. G., Leslie, A. G. W., and Walker, J. E. (2000) *Nature Struct. Biol.*, **7**, 1055-1061.
12. Hisabori, T., Ueoka-Nakanishi, H., Konno, H., and Koyama, F. (2003) *FEBS Lett.*, **545**, 71-75.
13. Bald, D., Noji, H., Stumpp, M. T., Yoshida, M., and Hisabori, T. (2000) *J. Biol. Chem.*, **275**, 12757-12762.
14. Hightower, K. E., and McCarty, R. E. (1996) *Biochemistry*, **35**, 4846-4851.
15. Ponomarenko, S., and Strotmann, H. (2003) in *The 7th Int. Congr. of Plant Molecular Biology*, Barcelona, S07-22.
16. Hisabori, T., Motohashi, K., Kroth, P., Strotmann, H., and Amano, T. (1998) *J. Biol. Chem.*, **273**, 15901-15905.
17. Ueoka-Nakanishi, H., Nakanishi, Y., Konno, H., Motohashi, K., Bald, D., and Hisabori, T. (2004) *J. Biol. Chem.*, **279**, 16272-16277.
18. Sambrook, J., Fritsch, E. F., and Maniatis, T. (1989) in *Molecular Cloning: a Laboratory Manual*, 2nd Edn., Cold Spring Harbor Press, NY.
19. Klionsky, D. J., Brusilow, W. S. A., and Simoni, R. D. J. (1984) *Bacteriology*, **160**, 1055-1060.
20. Sutcliffe, J. G. (1979) in *Cold Spring Harbor Symp. Quant. Biol.*, **43**, 77-90.
21. Iwamoto, A., Omote, H., Hanada, H., Tomioka, N., Itai, A., Maeda, M., and Futai, M. (1991) *J. Biol. Chem.*, **266**, 16350-16355.
22. Ponomarenko, S. (2004) *J. Biotechnol. Theory Practice*, **4**, 51-57.
23. http//www.qiagen.com
24. Tanaka, S., Lerner, S. A., and Lin, E. C. (1967) *J. Bacteriol.*, **93**, 642-648.
25. Schnick, C., Forrest, L. R., Sansom, M. S., and Groth, G. (2000) *Biochim. Biophys. Acta*, **1459**, 49-60.
26. Younis, H. M., Winget, G. D., and Racker, E. (1977) *J. Biol. Chem.*, **252**, 1814-1818.
27. Thompson, J. D., Higgins, D. G., and Gibson, T. J. (1994) *Nucleic Acids Res.*, **22**, 4673-4680.
28. Ponomarenko, S. V., and Ponomarenko, I. N. (1996) *Izv. MS-AS RK. Ser. Biol.*, **6**, 57-60.
29. Kayser, O. (2002) *Grundwissen Pharmazeutische Biotechnologie*, Verlag Teubner, Stuttgart.
30. Watts, S. D., Zhang, Y., Fillingame, R. H., and Capaldi, R. A. (1995) *FEBS Lett.*, **368**, 235-238.
31. Ponomarenko, S., Volfson, I., and Strotmann, H. (1999) *FEBS Lett.*, **443**, 136-138.
32. Andrews, S. H., Peskova, E. B., Polar, M. K., Herlihz, V. B., and Nakamoto, R. K. (2001) *Biochemistry*, **40**, 10664-10670.
33. Hausrath, A. C., Gruber, G., Matthews, B. W., and Capaldi, R. A. (1999) *Proc. Natl. Acad. Sci. USA*, **96**, 13697-13702.
34. Smart, E. J., and Selman, B. R. (1991) *Mol. Cell. Biol.*, **11**, 5053-5058.
35. Ivashchenko, A. T., Karpenyuk, T. A., Ponomarenko, S. V., Uteulin, K. R., Goncharova, A. V., Gabdulkhaeva, B. B., and Zakarina, A. E. (1992) *J. Evolut. Biochem. Physiol. (Russ. Acad. Sci.)*, **28**, 287-297.