Heterologous Overexpression of Membrane-Anchored Subunit II of Spinach Chloroplast ATP Synthase and Its Detergent-Free Purification as a Soluble Protein*

Hans-Jürgen Tiburzy, Martin Zimmermann, Regina Oworah-Nkru

and Richard J. Berzborn

Lehrstuhl für Biochemie der Pflanzen, Fakultät für Biologie der Ruhr-Universität Bochum, D-44780 Bochum, Germany

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Subunit II is one of the four nonidentical subunits of the membrane integral, proton-transporting moiety (CF0) of the chloroplast ATP synthase. In chloroplasts of spinach leaves, it is the only nuclear-encoded CF0 subunit. It has been deduced that CF0,II is not an additional subunit typical for photosynthetic organisms with no counterpart in E.coli, but equivalent to E. coli subunit b (Tiburzy, H.-J. and Berzborn, R. J. (1997), Z. Naturforsch. 52c, 789–798). Heterologous expression of subunit II was achieved by using the bacterial expression vector pT7-7. Recombinant subunit II (Iirec) does not integrate into the bacterial membrane nor does it precipitate into inclusion bodies. Gel filtration chromatography indicates that IIrec forms higher order aggregates. In three chromatographic steps approx. 10 mg of soluble IIrec of electrophoretic homogeneity are obtained from one liter of bacterial culture without using detergents. Thus, a eukaryotic membrane-anchored protein has been overexpressed in E. coli and has been purified in a soluble form.

Introduction

Subunit II is one of the four nonidentical subunits of the membrane integral, proton-transporting moiety (CF0) of chloroplast ATP synthase (Otto and Berzborn, 1989; Pancic et al., 1992; Herrmann et al., 1993). In chloroplasts of spinach leaves, subunit II is the only nuclear-encoded CF0 subunit (Nelson et al., 1980); it is cytoplasmatically synthesized as a precursor of 24.5 kDa and processed to a mature protein of 16.5 kDa (Herrmann et al., 1993). From analysis of 51 amino acid sequences of b-type subunits from chloroplasts of higher plants and algae as well as from nonphotosynthetic and photosynthetic eubacteria concerning similarities in primary structure, isoelectric point and a discovered discontinuous structural feature, it has been concluded (Tiburzy and Berzborn, 1997) that subunit II is the equivalent of subunit b of nonphotosynthetic eubacteria like E. coli and not subunit I, as formerly sug-

Abbreviations: CF0, membrane integral moiety (F0) of chloroplast ATP synthase; CF0/EF1, peripheral coupling factor (F1) of chloroplast/E. coli ATP synthase; IIrec recombinant form of subunit II; SDS-PAGE, sodium dodecyl sulfate polyacrylamide gel electrophoresis; Sarkosyl, N-Dodecanoyl-N-methylglycine (N-Lauroyl-sarcosine); ELISA, enzyme-linked immunosorbtent assay.


Reprint requests to Prof. Dr. R. J. Berzborn.

Fax: (+49) 234-7094322

E-mail: richard.j.berzborn@ruhr-uni-bochum.de

The precise role of subunit II within the holoenzyme, ATP synthase, is unknown. Its equivalence to E. coli subunit b suggests that subunit II is involved in binding of the peripheral CF₁ to the membrane integral part, CFₒ. This suggestion is supported by immunochemical data on in situ topography of subunit II: together with subunit I it extends into the stroma and is partially shielded within or below CF₁ (Otto and Berzborn, 1989). It remains to be established whether subunit II participates in building up the central stalk (Otto and Berzborn, 1989), which has been visualized by electron microscopy (Boekema et al., 1988), or in forming a second stalk functioning as a stator, as has recently been speculated for subunit b of E. coli (Wilkens et al., 1997), and which has been made visible by electron microscopy of detergent-solubilized and negatively stained ATP synthase of E. coli (Wilkens and Capaldi, 1998). In the case of E. coli, it has been demonstrated by cryoelectron microscopy that an N-terminal truncated and soluble subunit b forming a dimer does not bind to EF₁ at the center but seems to be located more at the periphery (Wilkens et al., 1994), indicating that subunit b may not be part of the central stalk.

For subunit II, however, a direct binding to CF₁ is uncertain. Cross-links of subunit II with CF₁ subunits α, β and γ, obtained from an isolated ATP synthase from Vicia faba, have been described (Süss, 1986). Because of a low yield of products, nonspecific cross-links and aggregation cannot be excluded (Feng and McCarty, 1990). In addition, the results were hard to interpret due to difficulties with nomenclature and identification of subunits (Berzborn et al., 1990; Wetzel and McCarty, 1993). Using the detergent Zwittergent 3–12, subunits I, II and IV have been dissociated from the isolated holoenzyme, CFₒCF₁. The capability of association of subunit II with CF₁ has been investigated after removing the detergent; from the failure of association it has been concluded that subunit II does not bind directly to CF₁ within CFₒCF₁ (Feng and McCarty, 1990). But this failure might be caused by residual amounts of detergent still bound to CF₁ (and/or subunit II) interfering with re-association. Since detergents cannot be avoided in the isolation of subunit II from thylakoid-bound ATP synthase, we intended to produce subunit II as a recombinant protein by heterologous expression.

Another open question is the stoichiometric amount of subunit II within the ATP synthase (van Walraven and Bakels, 1996; Nalin and Nelson, 1987). Previous estimations of radioactivity incorporated into thylakoid proteins in vivo revealed that the counts attributed to subunit I were half as those attributed to subunit II (Süss and Schmidt, 1982); this result probably gave rise to the conclusion (Wetzel and McCarty, 1993) that there might be one copy of subunit I and two copies of subunit II per ATP synthase complex. From the recently concluded equivalence of chloroplast subunit II and E. coli subunit b (Tiburzy and Berzborn, 1997), which is present in two copies (Foster and Fillingame, 1982), it is indeed suggested that also subunit II may exist in two copies. However, in isolated thylakoids, the radioactivity in a band after SDS-PAGE cannot be assigned to a single polypeptide; in addition, in the isolated ATP synthase complex, subunit II as well as subunit I were measured in substoichiometric amounts due to loss of these subunits during preparation. We intend to specifically quantify ATP synthase subunits in isolated thylakoids, especially subunit II, i.e. to develop an ELISA using heterologous expressed subunit II as a standard.

In the present paper, we report on the overexpression of subunit II in E. coli and its detergent-free isolation and purification in a solubel form. To our knowledge this is the first description of a recombinant eukaryotic membrane integral protein that is neither inserted into the bacterial membrane nor precipitated into inclusion bodies after overexpression in E. coli.

Materials and Methods

Chemicals and enzymes

Chemicals were of highest purity available. Restriction endonucleases and Klenow fragment were purchased from Boehringer (Mannheim), T₄ DNA ligase from GIBCO BRL, Sarkosyl (N-Lauroyl-sarcosine), lysozyme, DNase I and antibiotics from Sigma, α³⁵S]dATP from Amersham Buchler, TSK Butyl-650 (M) and TSK DEAE-650 (S) from Merck, Sepharacyl S-200 HR and Chelating Sepharose Fast Flow from Pharmacia, Coo- massie (Serva blue G-250) from Serva, PVDF-Im-
mobilon nitrocellulose membrane (0.45 μm) from Millipore, molecular weight standard proteins for SDS-PAGE from Fluka.

**Strains and plasmids, growth conditions**

*E. coli* strain DH5α carrying plasmid Bluescript M13(−)/II (with 589 bp insert encoding mature subunit II, kindly provided by Prof. Dr. R. G. Herrmann, University Munich) was grown in LB medium at 37 °C under ampicillin selection (50 μg/ml). *E. coli* strain TG1 carrying expression vector pT7-7 (Tabor, 1990) or expression vector pMZ1-1 (this work) was grown in LB medium at 37 °C under ampicillin selection (50 μg/ml). *E. coli* strain K38 carrying plasmid pGP1-2 (Tabor and Richardson, 1985) was grown in LB medium at 30 °C under kanamycin selection (50 μg/ml). For expression of the recombinant protein, *E. coli* strain K38 carrying both plasmids pGP1-2 and pMZ1-1 was grown in 2x YT medium with ampicillin and kanamycin (each 50 μg/ml) at 30 °C.

**Plasmid DNA purification, transformation of E. coli and DNA sequencing**

Purification of plasmid DNA after alkaline lysis (Birnboim and Doly, 1979) of bacteria and transformation of *E. coli* by plasmids using the calcium chloride method were done as described by Sambrook et al. (1989). Sequencing of single-stranded DNA isolated from vectors M13mp18/mp19 was performed with the TaqTrack Sequencing System from Promega based upon the dideoxy chain-terminating method of Sanger et al. (1977) using α[^35]S]dATP.

**Expression of IIrec**

For analytical preparation, 3 ml of 2× YT medium (with ampicillin and kanamycin, each 50 μg/ml) were inoculated with 200 μl of an overnight culture of *E. coli* strain K38 carrying both plasmids pGP1-2 and pMZ1-1 (K38/pGP1-2/pMZ1-1) and incubated for 4 h at 30 °C in a water bath with shaking. Induction of expression was achieved by raising temperature to 42 °C and continuing incubation at 42 °C in a water bath for 15 min. Subsequent growth was allowed at 37 °C for another 2 h. For SDS-PAGE or Western blot analysis, 200 μl of culture were centrifuged and the resulting sediment was dissolved in 100 μl of SDS-PAGE sample buffer. For large-scale preparation, 1.5 liters of 2× YT medium (with ampicillin and kanamycin, each 50 μg/ml) were inoculated with 50 ml of an overnight culture of *E. coli* strain K38/pGP1-2/pMZ1-1 and incubated at 30 °C in a water bath with shaking until an optical density (measured at 600 nm) greater than 1 was reached. Induction was achieved by stepwise addition (with shaking) of 2× YT medium pre-warmed to 90 °C until temperature was shifted to 42 °C (nearly 500 ml of 2× YT medium, monitored with a thermometer). Cells were left for 25 min at 42 °C. Subsequent growth was allowed at 37 °C for another 2 h. Cells were harvested by centrifugation (5,000×g, 15 min, 4 °C) and stored at −80 °C.

**Isolation and purification of IIrec**

Lysis: Frozen cells of induced *E. coli* strain K38/pGP1-2/pMZ1-1 obtained from one liter of culture were thawed at 4 °C for 1 h. For sonication, thawed cells were resuspended in 50 ml of ice cold lysis buffer (50 mM tris(hydroxymethyl)amino­methyl)-HCl, pH 8.0, 1 mM EDTA). Sonication were performed on ice with 60–80 watt pulses (Branson sonifier 250, maxi tip) in intervals of 15 seconds with 15 seconds rests between pulses. For lysozyme treatment, thawed cells were resuspended in 5 ml of ice cold lysis buffer. After addition of 10 mg of DNAse I and incubation on ice for 5 minutes, 250 μl of lysozyme solution (40 mg per ml distilled water) were added, and incubation on ice was continued for 1 h. Subsequently, the sample was diluted with 20 ml of ice cold distilled water.

Unbroken cells and cell debris were sedimented by centrifugation (27,000×g, 1 h, 4 °C). The supernatant was filter-sterilized (0.2 μm), adjusted to 50% ammonium sulfate saturation by adding one volume of neutralized saturated ammonium sulfate solution, and stored at 4 °C.

Since after sonication clearly less products of proteolysis of IIrec occurred when compared with the lysozyme treatment (as indicated by Western-blot analysis, data not shown), the former method was routinely used to lysate *E. coli*.

Chromatographic procedures: Chromatographic procedures were performed on an FPLC system (Pharmacia) equipped with a UV detector at room
temperature with filtered (0.45 μm) and degassed buffers.

Hydrophobic interaction chromatography (HIC): The ammonium sulfate precipitate resulting from one liter of bacterial culture (corresponding to 50 ml of sonicated cells) was dissolved in 50 ml of buffer A containing 10 mM Tris-HCl (pH 8.0), 60 mM ammonium sulfate and 2 mM EDTA. This solution was applied to a TSK Butyl-650 (M) column (1.6x11 cm) equilibrated with buffer A. The column was washed with buffer A until the UV absorption (280 nm) returned to its initial baseline. IIrec was eluted (i) with a 30-ml linear ammonium sulfate gradient from buffer A to buffer B (containing the components of buffer A, except of ammonium sulfate) followed by washing with 50 ml of buffer B, (ii) subsequently, with a 30-ml linear ‘water gradient’ from buffer B to distilled water followed by washing with 50 ml of distilled water.

Ion exchange chromatography (IEC): IIrec-containing peak fractions obtained by the ‘water gradient’ in HIC were combined, adjusted to 10 mM Tris-HCl (pH 8.0) and then pooled with IIrec-containing peak fractions obtained by the first gradient in HIC. This sample was applied to a TSK DEAE-650 (S) column (1x10 cm) equilibrated with buffer C containing 10 mM Tris-HCl (pH 8.0) and 40 mM ammonium sulfate. The column was washed with 30 ml of buffer C. To remove ammonium sulfate and to change the pH value, a 30-ml linear gradient from buffer C to buffer D (containing 10 mM Tris-HCl, pH 7.0) was applied followed by washing with 30 ml of buffer D. IIrec was eluted with a 30-ml linear sodium chloride gradient from buffer D to buffer E (containing 10 mM Tris-HCl, pH 7.0, 300 mM NaCl) followed by washing with 50 ml of buffer E.

Immobile ion metal affinity chromatography (IMAC): IIrec-containing peak fractions from IEC were combined and directly applied to a Chelating Sepharose Fast Flow column (1.6x6 cm) charged with Cu²⁺ (only the top 70% of the column) and equilibrated with buffer F containing 10 mM Tris-HCl (pH 7.0) and 150 mM NaCl. The column was washed with 2.5 column volumes of buffer F. IIrec was eluted with a 20-ml linear gradient from buffer F to buffer G (containing the components of buffer F with the addition of 200 mM NH₄Cl) followed by washing with 30 ml of buffer G.

Analytical methods

SDS-PAGE was carried out according to Lüntenberg et al. (1975) or according to Schägger et al. (1985). Proteins were stained with Coomassie. For protein sequencing, IIrec was blotted onto a PVDF-Immobilon nitrocellulose membrane (0.45 μm) according to the method of Dunn (1986). Amino-terminal protein sequencing of IIrec was performed by Dr. H. Meyer (Institut für Physiologische Chemie, Fakultät für Medizin, Ruhr-Universität Bochum) using a gas phase sequencer (Applied Biosystems 477 A).

Results

Construction of the expression vector pMZ1-1

For production of high amounts of subunit II in a form not denatured with treatment with detergents, a cDNA coding for the mature subunit II (kindly provided by Prof. Dr. R. G. Herrmann, University Munich, as a HindII/BamHI-fragment in plasmid Bluescript M13(−)) was cloned into the expression vector pT7-7 (Tabor, 1990) (Fig. 1). The HindII/BamHI-fragment differs from the original DNA sequence encoding the mature subunit II (Berzborn et al., 1990) in that it starts with the triplet GAC (coding for amino acid Asp) instead of GAA (coding for amino acid Glu). The resulting construct, referred to as pMZ1-1, should contain four additional codons upstream of the first codon of the sequence coding for the mature subunit II introducing four additional amino acids at the N-terminus of the protein to be expressed: Met (M), Ala (A), Arg (R) and Ile (I) (Fig. 1). The molecular weight of the recombinant protein should be 16,956 kDa as deduced from the sequence. Vector pMZ1-1 was transformed into E. coli strain K38/pGP1-2 leading to E. coli strain K38/pGP1-2/pMZ1-1. Transformants carrying vectors pMZ1-1 were identified by kanamycin/ampicillin selection followed by restriction analysis. As a control, E. coli strain K38/pGP1-2 was also transformed with plasmid pT7-7 (without insert) leading to E. coli strain K38/pGP1-2/pT7-7. To confirm that the complete DNA sequence of the HindII/BamHI-fragment was regained for expression of the entire subunit IIrec from vector pMZ1-1, the fragment was also cloned into M13mp18/mp19, and the DNA was sequenced.
expression vector pT7-7

5'...ATGGCTAGAATT...GGATCC...3'
3'...TACCGATCTTAAG...CCTAGG...5'

Bluescript M13(-)/II

5'...GTCGACGAAATCGAAGAGGTCC...GGATCC...3'
3'...CAGCTGCTTTAGCTTTCCGAGG...CCTAGG...5'

↓ 1. EcoRI
↓ 2. fill-in
↓ 3. BamHI

↓ HindII/BamHI

T4 DNA ligase

pMZ1-1

M A R I D E I E K A S...

5'...ATGGCTAGAATTGACGAAATCGAGAAAGCGTCC...GGATCC...3'
3'...TACCGATCTTAAGCTGCTTTAGCTTTCCGAGG...CCTAGG...5'

Fig. 1. Construction of vector pMZ1-1 from vector pT7-7 and cDNA encoding mature subunit II for expression of recombinant II (II_{rec}) in *E. coli*. Plasmid pT7-7 contains a unique BamHI site but lacks a HindII site in the polylinker region. To generate a blunt-ended terminus for proper insertion of the HindII/BamHI-fragment (bold letters), pT7-7 was first digested with EcoRI. In a second step, the resulting 5'-protruding ends were filled-in with Klenow fragment of *E. coli* DNA polymerase I. In a third step, the still linearized pT7-7 was digested with BamHI to create a cohesive terminus enabling complementary base pairing with the BamHI site on the HindII/BamHI-fragment. For re-isolation of the fragment from vector Bluescript, the isoschizomer HindII was used instead of HindII. Ligation of the HindII/BamHI-fragment to the modified pT7-7 vector was performed by using T4 DNA ligase.

The sequence obtained (not shown) is in full agreement with the sequence coding for the mature subunit II (Herrmann et al., 1993; Berzborn et al., 1990), including replacement of initiating codon GAA by GAC as mentioned above.

**Identification of II_{rec}**

Upon heat induction, strain K38/pGP1-2/pMZ1-1 produces an additional prominent protein visible on SDS gels when compared with control strain K38/pGP1-2/pT7-7 (Fig. 2); the protein migrates with an apparent molecular weight of about 17 kDa as to be expected from the deduced molecular weight of 16.956 kDa for II_{rec}. On Western blots, this protein was recognized by antibodies raised against subunit II isolated from spinach thylakoids (data not shown). The identity of the partially purified protein was further verified by automated Edman degradation which reveals the N-terminal amino acid sequence

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The comparison with the expected sequence (cf. Fig. 1) shows that the initiating methionine was
cleaved off. Thus, the overexpressed protein is identified as the recombinant form of spinach chloroplast subunit II, denoted as $\text{II}_{\text{rec}}$.

**Solubility of $\text{II}_{\text{rec}}$**

For isolation of $\text{II}_{\text{rec}}$, induced cells were either sonicated or treated with lysozyme. Unbroken cells and cell debris were sedimented by low speed centrifugation. Increasing amounts of $\text{II}_{\text{rec}}$ in the supernatant observable in SDS-PAGE correlated with an increasing degree of lysis as monitored by measurement of turbidity at 600 nm. This indicates that $\text{II}_{\text{rec}}$ does not precipitate into inclusion bodies. Even after high speed centrifugation of the supernatant at 100,000×g (1 h, 4 °C), $\text{II}_{\text{rec}}$ remains in the supernatant indicating that it is not inserted into the bacterial membrane. Thus, the recombinant form of subunit II, which is membrane-embedded in thylakoids, behaves like a soluble protein.

Size-exclusion chromatography (SEC) showed that purified $\text{II}_{\text{rec}}$ (17 kDa) does not migrate according to its molecular weight. When using a Sephacryl S-200 HR column (fractionation range 5–250 kDa), $\text{II}_{\text{rec}}$ eluted at an initial concentration of 0.2 mg/ml, elutes in the void volume. In the presence of the detergent Sarkosyl (0.5%), $\text{II}_{\text{rec}}$ is retarded, but migrates at an apparent molecular weight comparable to that of bovine serum albumin (67 kDa). These discrepancies between the molecular weight deduced from amino acid sequence and the apparent molecular weight in SEC – which were independent of the method of lysis – suggest that $\text{II}_{\text{rec}}$ aggregates forming oligomers, and, in addition, may be of elongated nature.

**Purification of $\text{II}_{\text{rec}}$**

Purification of $\text{II}_{\text{rec}}$ was achieved by three chromatographic steps. After hydrophobic interaction chromatography (HIC), an ion exchange chromatographic step was introduced to adjust the sample for immobilized metal ion affinity chromatography (IMAC). Since after centrifugation of the lysate, the majority of bacterial membrane proteins is removed from the supernatant, $\text{II}_{\text{rec}}$ can be separated from most soluble bacterial proteins due to its stronger hydrophobic interactions with the chromatographic matrix. Buffer conditions for adsorption of $\text{II}_{\text{rec}}$ were adjusted such that the bulk of E. coli proteins does not bind to the column (Fig. 3, peak D). Elution of $\text{II}_{\text{rec}}$ occurs in two peaks (Fig. 3, peaks 1 and 2); re-chromatography of each of them results again in two peaks. In subsequent purification steps, no differences between $\text{II}_{\text{rec}}$ from these two peaks were observed. Routinely, fractions of both peaks were pooled prior to further treatment. To remove ammonium ions and EDTA interfering with IMAC, the combined peak fractions from HIC were subjected to an ion exchange chromatography. Under the conditions employed, some additional resolution occurs (Fig. 4, lane 3). $\text{II}_{\text{rec}}$-containing peak fractions were combined and directly subjected to IMAC. To purify further, we made use of the fact that $\text{II}_{\text{rec}}$ does not contain any of the IMAC-relevant amino acids, histidine, cysteine or tryptophan (Porath et al., 1975). Adsorption of $\text{II}_{\text{rec}}$ to the IMAC column charged with Cu$^{2+}$ does occur, probably only via the free amino-terminal group as described for synthetic peptides (Hansen and Lindeberg, 1994). This binding is weaker than that via the IMAC-relevant amino acids. Therefore, $\text{II}_{\text{rec}}$ is eluted to a great extend with the ammonium chloride gradient in an electrophoretic homogeneous form as revealed by Coomassie-stain after SDS-PAGE (Fig. 5; Fig. 2, lane 3), while E. coli proteins and a residual amount of $\text{II}_{\text{rec}}$ remain bound to the column, which are eluted upon treatment with EDTA. The isolated $\text{II}_{\text{rec}}$ can be stored as ammo-

![Fig. 3. Purification of $\text{II}_{\text{rec}}$. Hydrophobic interaction chromatography of the E. coli K38/pGPl-2/pMlZ1-1 lysate on a TSK Butyl 650 (M) column (for details, see Materials and Methods). A: sample loading and washing. B: ammonium sulfate gradient. C: ‘water gradient’. D: peak of the unbound proteins. 1, 2: $\text{II}_{\text{rec}}$-containing peaks.](image-url)
ture after induction. The initial amount of II\textsubscript{rec} present in the bacterial cells before lysis appears to be considerably higher: approximately 50 mg/liter culture as estimated from comparison of Coomassie-stained bands after SDS-PAGE (cf. Fig. 2, lanes 2 and 3). Loss of II\textsubscript{rec} is e.g. due to incomplete lysis by sonication and to incomplete desorption in IMAC.

**Discussion**

In order to investigate the role of subunit II, the only nuclear-encoded CF\textsubscript{o} subunit in ATP synthases, e.g. its stoichiometry and its interactions with CF\textsubscript{1}, we intended to produce subunit II as a recombinant protein by heterologous expression in *E. coli*. From expression vector pT7-7 (Tabor, 1990), vector pMZ1-1 has been created encoding the mature subunit II, including replacement of the first residue Glu by Asp and with the addition of four amino acids (Met, Ala, Arg, Ile) at the N-terminus. The initiating Met is removed in *E. coli*. High-level expression of II\textsubscript{rec} (approx. 50 mg/liter of culture) in soluble form has been achieved. With essentially two chromatographic steps it is possible to obtain approx. 10 mg of II\textsubscript{rec} of electrophoretic homogeneity from one liter of culture.

The precursor of subunit II from spinach has already been synthesized in vitro (Herrmann *et al.*, 1993; Michl *et al.*, 1994). But heterologous expression in bacteria of nuclear-encoded chloroplast subunit II is demonstrated here for the first time. An attempt to investigate whether subunit II would be able to replace subunit b in *E. coli* (Schmidt, 1992) failed; there was no evidence provided for any expression of subunit II.

Noticeably, II\textsubscript{rec} can be isolated in a soluble form after overexpression in *E. coli*. This is not trivial for eukaryotic membrane proteins (Grisshammer and Tate, 1995; Evans *et al.*, 1995). The other eukaryotic membrane proteins successfully overexpressed in *E. coli*, are either integrated into the bacterial membrane or accumulated in inclusion bodies (Grisshammer and Tate, 1995; Evans *et al.*, 1995; Miroux and Walker, 1996). Even for subunit b, the equivalent of chloroplast subunit II in *E. coli* ATP synthase (Tiburzy and Berzborn, 1997), overexpression in a soluble form has only been achieved after removal of the N-terminal hydrophobic region (Dunn, 1992).
Heterologous Overexpression of Subunit II of Chloroplast ATP Synthase

II\textsubscript{rec} does not integrate into the bacterial membrane, probably because it lacks its N-terminal presequence containing a second hydrophobic stretch (Herrmann et al., 1993; von Heijne et al., 1989). Integration of spinach subunit II into thylakoid membranes is suggested to occur spontaneously in co-operation with the N-terminal presequence of its precursor, as proposed for M13 procoat protein in \textit{E. coli} (Michl et al., 1994). Furthermore, II\textsubscript{rec} contains an arginine in addition to the lysine in front of the hydrophobic stretch; for anchoring of II\textsubscript{rec} in the bacterial membrane \textit{via} the N-terminal hydrophobic stretch, these positively charged residues would have to be translocated across the membrane. But it has been found that residues Arg and Lys are avoided in translocated regions shorter than 70–80 residues (positive-inside rule, von Heijne and Gavel, 1988).

II\textsubscript{rec} does not precipitate into inclusion bodies. The formation of higher order aggregates, observed in size exclusion chromatography, is probably caused by hydrophobic interactions excluding the hydrophobic stretch from the aqueous environment and thus allowing II\textsubscript{rec} to keep soluble. This aggregation of II\textsubscript{rec} might be a drawback when analyzing interactions between II\textsubscript{rec} and CF\textsubscript{f}, since it may interfere with direct binding of II\textsubscript{rec} to isolated CF\textsubscript{f}. In preliminary studies, we were not able to observe a direct binding (data not shown). As a further consequence of aggregation and also of low solubility, for the time being, the structure of II\textsubscript{rec} cannot be analyzed by X-ray crystallography nor by NMR spectroscopy. For these purposes, it might be more advantageous to express subunit II without the hydrophobic stretch as it has been done for subunit b of \textit{E. coli} (Dunn, 1992). However, for quantitative determinations of subunit II by means of an ELISA, it is necessary to obtain an almost authentic subunit II. In a following paper, we will report on the development of an ELISA for estimation of the stoichiometry of subunit II; purified recombinant polypeptide II\textsubscript{rec} will be used as a standard for quantifying the content of subunit II in thylakoids.

Acknowledgement

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\textit{Note added in proof:} After demonstrating a second connection between CF\textsubscript{f} and CF\textsubscript{f} by selecting and averaging pictures of side views of detergent solubilized and negatively stained CF\textsubscript{f}/CF\textsubscript{f} preparations [B. Böttcher, L. Schwarz and P. Gräber (1998), J. Mol. Biol. 281, 757–762], investigations on location, structure and function of CF\textsubscript{f}/II are even more urgent.


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