The "Additional Subunit" CF_0II of the Photosynthetic ATP-Synthase and the Thylakoid Polypeptide, Binding Ferredoxin NADP Reductase: Are they Different?

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Dedicated to Professor Wilhelm Menke on the occasion of his 80th birthday

Amino Acid Sequence, Antibodies, Coupling Factor, Photophosphorylation

Evidence is presented to support the notion that the 16 kDa thylakoid polypeptide, called CF_0II, is an essential subunit of the photosynthetic ATP-synthase complex CF_0CF_1:

It is co-isolated with the other subunits of CF_0CF_1 in preparations either using octylglucoside/cholate or Triton X-100. It is co-precipitated by antibodies together with the other CF_0CF_1 subunits. It is immunochemically not related to thylakoid polypeptides of higher molecular weight nor to some thylakoid polypeptides with similar apparent molecular weight between 16 and 18 kDa: $CF_1\varepsilon$, CF_0I, subunit IV of the b_6f complex, the 16.5 kDa peripheral polypeptide of the oxygen evolving complex of PS II, and the intrinsic ferredoxin NADP reductase binding protein.

The N-terminal amino acid sequences of CF_0II and the reductase binding protein is determined by Edman degradation and compared: The two sequences are different and not identical to other characterized thylakoid polypeptides.

Monospecific antibodies against CF_0II inhibit rebinding of CF_1 to EDTA treated thylakoid membranes, H^+ efflux from EDTA treated membranes and cyclic photophosphorylation. Thus the additional polypeptide CF_0II qualifies for a functional subunit of the photosynthetic ATP-synthase.

Introduction

The intricate inner membrane structure of chloroplasts has been named *thylakoids* by W. Menke in 1961 [1], recognizing their sacklike structure and drawing the attention to this significant compartimentation within the chloroplast. Photosynthetic water oxidation and NADP reduction and coupled ATP-synthesis is energized by light and catalyzed by four large protein complexes embedded in these thylakoid membranes. It is assumed that any protein subunit can only be a functional subunit of *one* of these complexes; it is also assumed that during separation and isolation of these complexes for biochemical analysis no loss or artificial interchange of subunits or contamination with foreign chloroplast polypeptides occurs.

In crude preparations of CF_0CF_1, the photosynthetic ATP-synthase complex, besides high molecular weight aggregates, the large and the small subunits of the ribulose-bisphosphate carboxylase, the ferredoxin-NADP reductase and some LHCP (27 kDa) are observed on SDS polyacrylamide gels. Upon removal of the contaminations, partially identifiable by specific antibodies, nine different polypeptides remain and seem to constitute the photosynthetic ATP-synthase [2–7]. By compari-

Abbreviations: CF_1, peripheral moiety of chloroplast ATP-synthase; CF_0, membrane integral moiety of chloroplast ATP-synthase; F_1, peripheral moiety of ATPase of oxidative phosphorylation; F_0, membrane integral moiety of ATPase of oxidative phosphorylation; α, β, γ, δ, ε, subunits of CF_1 or F_1; IV, I, II, III, subunits of CF_0; a, b, c, subunits of F_0; Chl, chlorophyll; ELISA, enzyme-linked immunosorbent assay; PMS, phenazine methosulfate; 278-0, preimmune serum of rabbit 278; 278-1,2,..., successive bleedings of *anti*-CF_0II serum; SDS-PAGE, polyacrylamide gel electrophoresis in the presence of sodium dodecylsulfate; PTH, phenyl thiohydantoin.

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son with the composition of the peripheral CF_1 moiety with its subunits α, β, γ, δ and ε [8], the other polypeptides I, II, III and IV are concluded to be subunits of the membrane integral CF_0 [2, 3, 9].

The F_0F_1 ATPase from *E. coli* consists of eight subunits [10]. From amino acid sequence homologies and similarities in the hydropathy profils follows: Chloroplast CF_0IV corresponds to *E. coli* F_0a, CF_0I to F_0b and CF_0III to F_0c; thus CF_0II apparently is an "additional" polypeptide in preparations of the photosynthetic ATP-synthase [2–7 and reviews, 11]. The question arises whether it is an essential and functional subunit of the ATP-synthase and different from all characterized thylakoid polypeptides or a contamination in the preparation.

Since in crude preparations of CF_0CF_1 the ferredoxin NADP reductase is always found (running just above CF_1 gamma on SDS gels) and an integral reductase binding protein with an apparent molecular weight of about 17 kDa is shown to exist in thylakoids [12, 13], the 16.5 kDa so-called CF_0II could *e.g.* be identical to this binding polypeptide.

In this publication we show: The polypeptide CF_0II with an apparent molecular weight between 16 and 18 kDa (depending on the gel system), co-isolates with the CF_0CF_1 complex also if for desintegration of the membrane Triton X-100 is used instead of β-octylglucoside; CF_0II is co-precipitated together with the other subunits of the ATP-synthase complex by antibodies; CF_0II is immunochemically unrelated to any thylakoid polypeptide of higher molecular weight; it is immunochemically also not related to some thylakoid polypeptides with similar molecular weight: CF_1 subunit ε, CF_0 subunit I, the peripheral 16.5 kDa subunit of the oxygen evolving complex of PS II, the 17.5 kDa subunit IV of the b_6f complex and not to the thylakoid polypeptide that binds ferredoxin NADP reductase.

Our determinations of the N-terminal amino acid sequences of CF_0II and of the reductase binding protein, reported here for the first time, confirm this conclusion. CF_0II is also different from CF_0IV [9], both in primary structure and in its genetic origin [5]. The N-terminal sequence of CF_0II proves that this additional polypeptide in CF_0CF_1 is also not identical to the 16 and 18 kDa polypeptides of PS I [14, 15].

We show that antibodies against CF_0II inhibit rebinding of CF_1 to EDTA treated thylakoids, H^+ efflux from EDTA treated thylakoids and PMS mediated cyclic photophosphorylation. We therefore conclude that CF_0II is an essential and functional subunit of the chloroplast ATP-synthase complex.

Results

Identity of CF_0 polypeptide II

a) Definition of CF_0II

The enzyme complex from chloroplasts, that catalyzes photosynthetic ATP-synthesis, can be isolated after dissolving the thylakoid membrane with the detergents β-octylglucoside and cholate [2] or Triton X-100 [3, 4]. Besides the subunits of CF_1 the CF_0CF_1 complex seems to contain four additional polypeptides. Although noticed in the initial publication, the uppermost of the additional bands was for several years disregarded as a contamination due to its poor stainability with Coomassie or Amido black. The others have been numbered I, II, III according to decreasing apparent molecular weight on SDS-PAGE. Polypeptide CF_0II has the property to migrate on SDS gels very close to $CF_1ε$ at about 16 kDa, but to change its apparent molecular weight to about 18 kDa and to migrate close to CF_0I in SDS gels with 6 M urea [5]. This polypeptide will be further characterized in this publication.

b) CF_0 polypeptide II belongs to the CF_0CF_1 complex

The thylakoid polypeptide CF_0II, as defined above, is seen in preparations of CF_0CF_1 after use of β-octylglucoside/cholate to separate the four large photosynthetic complexes [2, 5–7]. We developed an alternative method for isolation, using the less expensive Triton X-100 instead ([4], cp. Methods). On SDS polyacrylamide gels of the purified CF_0CF_1 complex nine polypeptides are resolved; apparent molecular weights and staining intensity of the bands are identical, when compared to the preparation using octylglucoside (Fig. 1a, b). Also in our preparation subunit CF_0IV is only seen as a shadow band after Coomassie staining, but present: It can be stained by silver according to Oakley *et al.* [16] and migrates

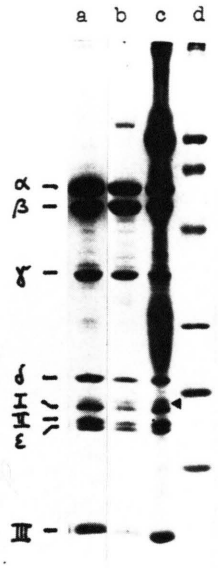

Fig. 1. Analysis of different preparations of CF_0CF_1 by SDS-PAGE. a) CF_0CF_1 complex isolated in Triton X-100, incl. sucrose density centrifugation (cp. Methods); b) CF_0CF_1 complex isolated in octylglucoside/cholate, incl. sucrose density centrifugation, according to [2]; c) CF_0CF_1 precipitated after isolation in Triton X-100 by antiserum 155 against CF_1; d) markers. The protein complexes were incubated in sample dissolving buffer for 1 h at 22 °C; CF_0CF_1 after octylglucoside isolation (b) shows aggregation of CF_0III at about 100 kDa [57], not however after isolation in Triton (a); 13–18% gradient of acrylamide, 2.5 M urea); triangel indicates shadow band, later identified as CF_0IV.

in an identical position compared to CF_0IV in a preparation [6] from the laboratory of P. Gräber, Berlin, which was generously sent to us (data not shown). Polypeptide CF_0II was always present and is migrating close to $CF_1\varepsilon$ without urea on the SDS gels, and close to CF_0I in the presence of urea also after isolation of the complex using Triton X-100.

Next the CF_0CF_1 complex, prepared in Triton X-100, was precipitated by an antiserum against CF_1, not containing any antibodies crossreacting with CF_0 polypeptides in Western blot. Upon SDS-PAGE of the precipitate the characteristic nine polypeptides of CF_0CF_1 were resolved, and in addition the light and heavy chains of the immunoglobuline (Fig. 1 c).

To further show that polypeptide CF_0II is tightly bound to CF_1, a sandwich-ELISA was carried out (Fig. 2): After incubation of the ELISA plates with catcher antibody (1), (antiserum 278 against electroeluted CF_0II, cp. Methods, dilution 1:25,000), and coating the remaining plastic with gelatine (2), a preparation of CF_0CF_1 (3), (prepared in Triton X-100, 1 µg in coating buffer) was added and excess removed by washing; as indicator antibody (4) the monoclonal mouse antibody 2C3 against $CF_1\beta$ was added, incubated and excess removed by washing; and finally the horseradish peroxidase conjugated 2nd antibody (goat anti mouse, dil. 1:2000) was added (5). The complete system resulted in an $OD_{450\,nm}$ of 1.1 after 10 min reaction time (6); with CF_1 as antigen or without serum 278 the color development was below 0.01, *i.e.* CF_0 does not bind to the gelatine coated plastic without the catcher antibody, serum 278 does not react with CF_1 and CF_0II indeed is tightly connected to $CF_1\beta$ under the incubation conditions.

c) CF_0 polypeptide II is immunochemically not related to another thylakoid polypeptide

In preparations of CF_0CF_1 two (or three) bands, migrating on SDS gels between $CF_1\delta$ and $CF_1\varepsilon$, have been seen after isolation from chloroplasts from spinach [2, 4–7], *Vicia faba* and *Avena sativa* [3] and lettuce (*Lactuca sativa,* var. Romaine) [17]. To show more specifically the presence of polypeptide CF_0II in thylakoids and CF_0CF_1 preparations in addition to CF_0I, the monospecific antisera 278

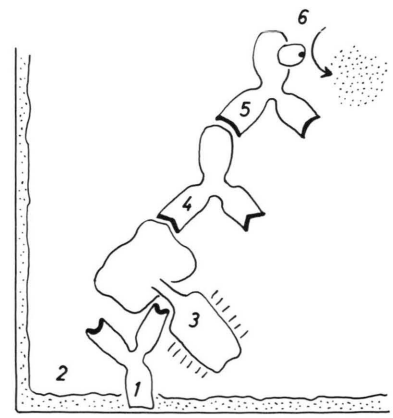

Fig. 2. Scheme of sandwich-ELISA to show that CF_0 polypeptide II and CF_1 subunit β are physically connected within the same protein complex (for procedure see text).

against spinach CF_0II (cp. Methods) and 313 against spinach CF_0I were used: On Western blots of isolated thylakoids bands of apparent molecular weight of 16 and 18 kDa were decorated, respectively, indicating the presence of polypeptides CF_0II and CF_0I in chloroplasts of *Spinacia oleracea, Pisum sativum, Zea mays, Sorghum bicolor, Lemna gibba* and *Chlamydomonas reinhardii* (data not shown).

From the fact that only one band is decorated with serum 278 against CF_0II on Western blots with isolated thylakoids we conclude that CF_0II is a polypeptide of its own identity and not a degradation product of any thylakoid protein of higher molecular weight, and that the precursor of nuclear encoded CF_0II with a molecular weight of 26 kDa [5] was not present in sufficient amounts to be detectable.

Then the immunochemical crossreactions of some thylakoid polypeptides with molecular weights of 16–18 kDa have been tested (Table I). The results shown depend on the specificities of the antisera, which in turn are dependent on the rabbits and the purity of the injected antigens, and also may depend upon the conformation of the test antigens; the conclusions are clear cut, however: Polypeptide CF_0II is immunochemically not related to the reductase binding protein (cp. also next chapter), to CF_0 subunit I, the 16.5 kDa peripheral protein of the oxygen evolving complex of PS II, subunit IV of the b_6f complex, CF_1 subunit ε and the small subunit of the ribulose-bisphosphate carboxylase.

If compared to thylakoids, PS II particles [18] or isolated b_6f complex [19], polypeptide CF_0II is always enriched in CF_0CF_1 preparations. Subunit IV of the b_6f complex, the reductase binding protein and the peripheral 16.5 kDa polypeptide of PS II are not detected in our preparations of CF_0CF_1. No crossreaction between the reductase binding protein (cp. next chapter), the 16.5 kDa peripheral protein of PS II and subunit IV of the b_6f complex, was observed, respectively.

d) N-terminal amino acid sequence of CF_0 polypeptide II is distinct

The clearest biochemical identification of a thylakoid polypeptide would be the determination of its amino acid sequence after purification. Reading frames on the chloroplast DNA became available coding for CF_0III, homologous to *E. coli* Fo c [20], and for CF_0I, homologous to *E. coli* Fo b [21, 9]; by chemical N-terminal sequencing the processing of CF_0I was determined in spinach [21]. Another reading frame was sequenced in the chloroplast ATPase operon, encoding for a hydrophobic polypeptide with some homologies to *E. coli* Fo a and called CF_0IV from spinach [9] and other species (for review cp. Hudson *et al.* [11]). Thus the nuclear encoded polypeptide CF_0II, if it can be shown to be an essential subunit of the chloroplast

Table I. Analysis of immunochemical cross reaction in Western blot of some thylakoid polypeptides and of cross contaminations in isolated complexes.

Immunogen-injected	Apparent molecular weight	Antiserum number	Strength of reaction with antigens blotted onto nitrocellulose after separation by SDS-PAGE								
			Reductase-binding protein	CF_0CF_1* total	CF_0CF_1 I	CF_0CF_1 II	ε	OEC 16.5	b_6f* total	SSU in crude CF_1	Thylakoids*
Reductase-binding protein	18.5	348	+++	–	–	–	–	–	–	–	++
CF_0I	18	313	–	+++	+++	–	–	–	–	–	++
OEC periph.	16.5	180	–	–	–	–	–	+++	–	–	++
Sub. IV b_6f	16	327	–	–	–	–	–	–	++	–	+
CF_0II	16/18	278	–	+++	–	+++	–	–	±	–	++
$CF_1\varepsilon$	16	272	–	++	–	–	+++	–	–	–	++
Small subunit of RuBP carb.	14	124	–	±	–	–	–	–	–	++	+

* On thylakoids or enriched complexes after SDS-PAGE: Strength of reaction in the respective molecular weight region.

ATP-synthase, would be the additional, ninth subunit which constitutes part of CF_0.

Therefore we have sequenced the isolated polypeptide II in CF_0 [22]: On analytical and preparative SDS gels according to Lugtenberg without urea a rather broad band of comigrating $CF_1\varepsilon$ and CF_0II was often observed. From Western immunoblots $CF_1\varepsilon$ appears to migrate mostly in the lower portion of the band, polypeptide II in the upper half. To avoid contact of polypeptide II with urea during preparation for automated Edman degradation it was not purified by rechromatography on gels with urea after electroelution from SDS gels without urea, as in the case of preparation of the antigen to be injected into rabbits or mice [23]; the upper part of the band was electroeluted; variant amounts of contaminating $CF_1\varepsilon$ were taken into account during the interpretation of the chromatograms of the PTH derivatives of the respective cycles. In the first analysis of the N-terminal sequence of CF_0II 16 residues could be identified in 20 cycles; in the second sequencing with an initial yield of 150 pmol the following 26 residues of CF_0II were identified:

X E I (L) K A S L F D_{10}
X N L T L P I I M A_{20}
E F L X L M

In the third sequencing of a new preparation of electroeluted CF_0II with an initial yield of 110 pmol less overlap of cycles was observed, but two residues raised in each cycle, one of which was identical to the sequence of $CF_1\varepsilon$ [24]. Sorting out these peaks the following residues can be assigned to CF_0 polypeptide II:

E E I E K A S L F D_{10}
F N L T L P I I M A_{20}
E F L F L M F A L D_{30}
K I

Residue Glu_4 is a correction of residue Leu_4 reported in the earlier communication [22]. The sequence was new, *i.e.* not found in the EMBL protein data bank, and proves biochemically that polypeptide CF_0II has its own identity and is not related to any characterized and sequenced chloroplast polypeptide. The first ten residues are identical to the 10 published amino acids, deduced from cDNA sequencing, following the completely published presequence of the precursor [25] with an apparent molecular weight of 26 kDa [5].

As mentioned [22] 19 hydrophobic residues are found between Asp_{10} and Asp_{30}, except for Glu_{21}, very suitable to build a membrane crossing span.

The sequence of $CF_1\varepsilon$ in this analysis started with

T L N L X V L T P N_{10};

i.e. subunit $CF_1\varepsilon$ had lost its initial Met [24].

Also the subunit CF_0I was electroeluted from preparative SDS gels after separation of CF_0CF_1, prepared in the presence of Triton X-100, and sequenced by automated Edman degradation. The analysis of the PTH derivatives confirmes biochemically the sequence of spinach CF_0I deduced from cDNA [9] up to residue Asp_{34}, and confirmes the findings that mature spinach CF_0I starts with G S F G and that the mRNA of the two exons is spliced to yield G_{30} V L S D_{34} etc. [21] (cp. Table IV).

Relation of polypeptide CF_0II and the thylakoid intrinsic reductase binding protein

Although polypeptide CF_0II is shown above to be immunochemically unrelated to thylakoid polypeptides with different molecular weight, and unrelated to some polypeptides with similar molecular weight, and although the N-terminal sequence of spinach CF_0II, as determined by us, is not identical or homologous to any polypeptide in the EMBL data bank, a mixup with other chloroplast polypeptides, not yet sequenced, with comparable apparent molecular weight cannot be excluded.

Since routinely some ferredoxin-NADP reductase is co-isolated with CF_0CF_1, it seemed worthwile to consider the reductase binding protein [12, 13]. We have purified this protein, produced and used antibodies against it and determined the N-terminal sequence. This polypeptide will now be shown to be different from CF_0II:

a) Production and properties of antibodies against the reductase binding protein

Monospecific antisera against the thylakoid intrinsic polypeptide binding ferredoxin-NADP reductase [12] have been prepared [26]. The isolation and characterization of the polypeptide will be described elsewhere (R. J. Berzborn and St. Schünemann, to be publ.). In principle the antigen for injection was isolated by the following procedure:

Spinach thylakoids were dissolved in Triton X-100, lipids removed by 25% ammonium sulfate, and the complex of ferredoxin-NADP reductase with its binding protein, stabilized by $MgCl_2$, purified by affinity chromatography on Blue Sepharose CL-6 B and anion exchange chromatography on DEAE Sepharose CL-6 B; the presence of reductase in the fractions was monitored by specific antibodies. Free reductase was separated from the complex on Sephadex S-200. The 16.5 kDa polypeptide of the oxygen evolving complex of PS II was identified by specific antibodies and also removed.

The binding protein, co-eluting with reductase, was separated by preparative SDS-PAGE and electroelution of a band of apparent molecular weight of about 18 kDa, as compared with $CF_1\delta$ (21 kDa) and $CF_1\varepsilon$ (16 kDa). Immunization of 2 rabbits (Nr. 347 and 348) was done with 80 µg protein for each treatment (cp. Methods).

On Western blots with the crude preparation of the complex of binding protein with reductase the antisera from both rabbits decorated only one band at 18 kDa, as did serum 348 on thylakoids; serum 347 was negativ on blots with thylakoids. In double diffusion tests according to Ouchterlony one precipitation line with dissolved thylakoids or crude preparations of binding protein precipitated, but no cross reaction occurred with reductase or other isolated proteins (data not shown).

Antibodies seem to dissociate the complex of binding protein and reductase, since it was not possible to demonstrate a "line of identity" with anti reductase, anti binding protein and the complex, or to co-precipitate the two proteins with either antiserum. We are sure to have isolated the reductase binding protein, however: It is present in preparation according to Vallejos *et al.* [12], although not identical to the 16.5 kDa peripheral PS II polypeptide of the oxygen evolving complex present in that preparation. In the presence of $MgCl_2$ it is co-eluted together with reductase in all steps of our preparation; it forms a crosslink upon glutaraldehyde treatment, positive on Western blots with *anti*-reductase at about 60 kDa; it does not pass through a Amicon YM 30 filter in the presence of $MgCl_2$, but after dissociation of the complex with reductase by EDTA (data not shown). As reported in Table I the antisera 347 and 348 against this polypeptide did not crossreact on Western blots with the thylakoid polypeptides of similar molecular weight, tested, especially not with CF_0 polypeptide II.

b) N-terminal sequence of the reductase binding protein

The reductase binding protein, isolated in denatured conformation by electroelution from SDS gels and used for immunization, was sequenced by automated Edman degradation. In the first run the N-terminal residues

A V A M X T S Q P S

could be identified with an initial yield of 32 pmol. In a second preparation, not used for immunization, two residues could be identified in each cycle. Using the ten residues of the first analysis, the contamination could be found to be the L_{12} protein from ribosomes with the sequence A V E A P E K I E Q etc. Taking this sequence into account, the following residues with an initial yield of 120 pmol could be assigned to the reductase binding protein:

A V A M D T S Q P S_{10}
P S S D Q D Q T x x_{20}

This sequence is not identical or homologous to any sequenced polypeptide (Swiss Prot. rel. 12), especially not to any sequenced thylakoid polypeptide (cp. Table IV), *i.e.* in particular this polypeptide, co-eluting together with reductase is not a degradation product of reductase. The sequence supports the conclusion from immunochemical data that the reductase binding protein is not related to the CF_0 polypeptide II.

CF_0II is a functional subunit of the chloroplast ATP-synthase

a) Topography of polypeptide CF_0II

In a recent publication [23] we have shown that isolated spinach chloroplast thylakoids adsorb antibodies against CF_0 subunit I and polypeptide II; *i.e.* CF_0I and CF_0II are partially exposed at the matrix side. The adsorption capacity towards both antisera was increased by removal of CF_1 by EDTA and decreased again by rebinding of CF_1 [23]. Therefore CF_0II and CF_0I are partially shielded against antibody approach underneath CF_1. A

similar conclusion has been drawn earlier from similar results [4, 27].

Here we present further evidence for the location of a large proportion of CF_0II (and of CF_0I) on the matrix side of the thylakoid:

Polypeptide CF_0II is not extracted from the thylakoid membrane by EDTA treatment that resolved CF_1 (Table II), not even by NaBr treatment (data not shown). Polypeptide CF_0II is protected against degradation by trypsin *in situ* on the thylakoid membrane, since removal of CF_1 increases the susceptibility to trypsin (Table II). The same was found for CF_1I [28].

After trypsination the binding capacity of EDTA treated thylakoids for CF_1 is decreased (Table II). Since CF_0 subunit III is not degraded under these conditions (R. J. Berzborn and M. Schmidt, unpubl.) there may be a correlation between digestion of CF_0II and/or CF_0I and the loss of CF_1 binding capacity, but we do not have investigated CF_0IV.

b) Effects of monospecific antisera against CF_0II on photosynthetic reactions

– Antibodies produced by absorption from sera against CF_0CF_1

Antibodies against subunits CF_0I and CF_0III and polypeptide CF_0II have been produced in several rabbits by injecting the entire CF_0CF_1 complex after isolation in Triton X-100 and additional precipitation with an antiserum against CF_1 [4]. When these sera were absorbed with CF_1, in serum 187 only antibodies against CF_0 polypeptide II remained and a low titer in non absorbable antibodies against $CF_1\beta$ and $CF_1\gamma$, visible in Western blots after SDS-PAGE of CF_0CF_1.

This absorbed serum 187A inhibited rebinding of CF_1 to EDTA treated membranes [4] as determined quantitatively by rocket immunoelectrophoresis [29] of CF_1 in the supernatants; PMS-mediated cyclic photophosphorylation was inhibited up to 95%, when concentrated immunoglobuline from the serum 187A was used [4]; the globuline from controlsera had no effect. To exclude the possibility that the inhibition was caused by the non absorbable antibodies against $CF_1\beta$ or $CF_1\gamma$, the incubation was done in the presence of excess soluble CF_1 (titrations not shown here).

The absorbed serum 187A also inhibited H^+ efflux from EDTA treated thylakoids (Fig. 3). The control serum had no effect; the antibodies against CF_0II had no effect on the extend of Δ pH in con-

Table II. Residual amounts of $CF_1(\alpha)$ and $CF_0(II)$ after proteolytic treatment and subsequent reconstitution with CF_1 (normalized to amounts in untreated thylakoid membranes). After trypsination for 10 min at room temperature trypsin inhibitor was added, the samples were washed, reconstituted with CF_1 [47], washed again, dissolved with Triton X-100 (final conc. 0.5%) and diluted with carbonate buffer for coating; the amounts of antigen were determined in ELISA (cp. Methods) with the *anti*-$CF_1\alpha$ serum 250 (dil. 1:15,000) and the *anti*-CF_0II serum 278 (dil. 1:10,000).

Membrane type	µg Trypsin used/mg Chl	µg CF_1 added/mg Chl	% Residual and/or rebound CF_0II	$CF_1\alpha$
Thylakoids	–	–	100	100
Thylakoids	12	–	95	70
Thylakoids	1200	–	54	11
EDTA-treated	–	–	105	17
EDTA-treated	12	–	23	11
EDTA-treated	1200	–	4	4
EDTA-treated	–	110	96	30
EDTA-treated	12	110	27	10
EDTA-treated	1200	110	6	3
EDTA-treated	–	660	97	102
EDTA-treated	12	660	29	24
EDTA-treated	1200	660	5	3

trol thylakoids (Fig. 3), *i.e.* they do not uncouple. A similar absorbed antiserum (169 A), containing antibodies against CF_0I, II and III even stimulated residual photophosphorylation in EDTA-treated thylakoids [4, 27], *i.e.* reconstituted structurally like an energy transfer inhibitor (titrations not shown here).

– Antibodies produced with electroeluted CF_0II

By injection of electroeluted CF_0II antibodies against homogeneous but denatured CF_0II were produced that proved to be monospecific (serum 278, cp. Methods). Also these antibodies inhibited CF_1 rebinding to EDTA treated thylakoids (Table III). In this experiment the residual and rebound CF_1 on the thylakoid was determined quantitatively in ELISA.

Since the strength of the decorated band in Western blot did not decrease much upon absorption of this serum with thylakoids, *i.e.* CF_0CF_1 *in situ,* the titer of antibodies against the denatured CF_0II crossreacting with the native conformation of the exposed part of CF_0II in the presence of CF_1 was low. In accordance with this agglutination of thylakoid suspensions with serum 278 was only observed down to a dilution of 1:32; after CF_1 removal by EDTA the titer increased to 1:128 [28], which is still low compared to agglutination with *anti*-CF_1 that can be clearly seen at dilutions of 1:512 or more. Proton flux measurements were not investigated with this serum; the PMS-mediated cyclic photophosphorylation was inhibited significantly, up to 26% of the control values (data not shown).

Table III. Inhibition of rebinding of CF_1 to EDTA-treated thylakoids by antibodies against CF_0II and CF_0I. (The residual and rebound amounts of CF_1 were determined in ELISA with *anti*-$CF_1\alpha$ serum 250, as in Table II; all membranes were washed before CF_1 determination.)

Membrane type	% CF_1 (α)
Thylakoids	100
EDTA-treated	12
– and incubated with 16 µg CF_1/25 µg Chl	84
– and incubated with IgG from preimmune serum (6.4 µg/80 µg Chl), washed, and incubated with 16 µg CF_1/25 µg Chl	70
– and incubated with IgG from *anti*-CF_0II (278) (2.5 µg/80 µg Chl), washed, and incubated with 16 µg CF_1/25 µg Chl	20
– and incubated with IgG from *anti*-CF_0I (313) (6.4 µg/80 µg Chl), washed, and incubated with 16 µg CF_1/25 µg Chl	25

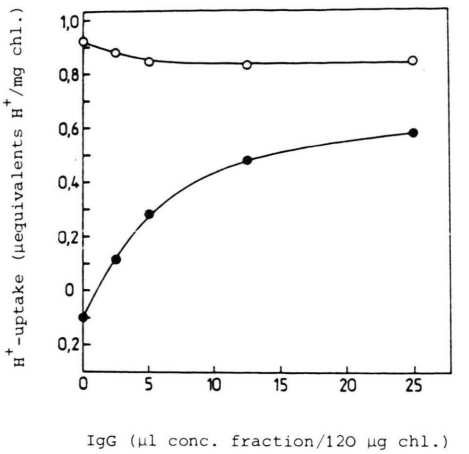

Fig. 3. Stimulation of extend of ΔpH in EDTA-treated thylakoids due to inhibition of H^+ efflux by antiserum 187 A against CF_0II; cp. text. Thylakoids were incubated for 5 min at 15 °C in the dark with concentrated immunoglobuline; 50 mM NaCl, 40 µg Chl/ml, 0.01 mM PMS, 0.2–0.5 mM tricine-NaOH, pH 7.2; then the pH was titrated to 6.5 with 0.01 N HCl and the H^+ uptake measured upon illumination; filter: RG 610 nm, Schott.

Discussion

The photosynthetic ATP-synthase complex CF_0CF_1 contains nine different polypeptides with a proposed stoichiometry of α_3, β_3, γ, δ, ε, I_{1-2}, II_{1-2}, III_{6-12} and IV. Since CF_0IV corresponds to *E. coli* F_0a [9], CF_0I to F_0b [21] and CF_0III to F_0c [20], what the amino acid sequence, the hydropathy profile, the apparent molecular weights and the position of the gens in the ATPase operon is concerned (Hudson and Mason, 1988), CF_0 polypeptide II seems not to correspond to any F_0 subunit, *i.e.* it may not be essential for the basic function of the ATP-synthase. These doubts are strengthened by the finding that CF_0I alone can substitute in function for F_0b in an *E. coli* mutant with deleted F_0b [30].

On the other hand a gen doublication of F_0b has been detected in the ATPase operon of cyanobacteria [31], and we found that the N-terminal se-

quence of CF_0II ([22] and this paper) can be aligned with the open reading frame F_0b', like the sequence of mature CF_0I with the reading frame F_0b (Table IV a). The number of identical residues and conservative replacements in the respective N-terminal sequences, all suitable to build transmembrane spans, indicate that CF_0II is homologous to F_0b' of cyanobacteria, and CF_0I to F_0b; both chloroplast polypeptides, however, seem equally homologous, or better equally *different* from *E. coli* F_0b (Table V). In cyanobacteria the expression of the reading frames, *i.e.* the existence of both polypeptides b and b′ in the ATP-synthase has not yet been demonstrated; it would be intriguing to speculate that during evolution from heterotrophs to autotrophs the function(s) of two copies of F_0b were distributed among two different subunits providing the opportunity to optimize the function of either one, *e.g.* subunit interactions within the CF_0CF_1 complex.

Table IV. a) Alignment of N-terminal amino acid sequences of CF_0 subunit I (this paper) and polypeptide CF_0II ([22] and this paper) from *Spinacia oleracea* and subunit F_0b from *E. coli* [52] and PS 3 [53] with homologous sequences deduced from open reading frames for F_0b and b′ from *Synechococcus* sp. 6301 [50] and *Anabaena* sp. 7120 [51]. b) Comparison of aligned CF_0 subunits with sequenced spinach thylakoid polypeptides of similar molecular weight: Ferredoxin-NADP reductase binding protein, as sequenced in this paper; mature CF_0IV ([6, 9], with corrections of printing errors in both publications, span not aligned); subunit IV of b_6f complex [54] (span not aligned); mature 16.5 kDa polypeptide of oxygen-evolving complex of PS II [55]; 16 (10.8) kDa subunit of PS I from barley [14]; 18 (20) kDa subunit of PS I [15]; CF_1 subunit ε [24].

a)

```
CFo II          EEIEKASLFDFNLTLPIIMA-EFLFLMFALD----KI....
Syn b'       ...VQEAEGGLFDLDATLPL-MAVQILVLVFLLNAVFYK----P
Ana b'       ...KVAKEGGLFDLDATLPL-MAIQFLLLALILNATLYK----P

E.c.b                  VNLNATI-LGQAIAFVLFVLFCM----KYVWPP
PS3 b                EAAHGISGGTI-IYQLLMFIILLALLR----KFAWQP

CFo I              GSFGFNTDILAT-NLINLSVVLPVLIFFG----KGVLSD
Syn b           ...SGFGLNLDLFET-NLINLAIIIGLLVYAG----RGFLGN
Ana b           ...GGFGLNTNILDT-NLINLAIIITVLFVFG----RKFLGN
```

b)

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r b p           AVAMDTSQPSPSSDQDQTxxxxxxxxxxxxxxxxxxxxxxx
CFo IV          GVEVGQHFYWQIGGFQIHDKALITSWVVIAILLGSAAIAVR
IV b6f          MGHNYYWPNDLLYIFPVVILGTIACNVGLAVLEPSNIGEPA
16.5 OEC        EARPIVVGPPPPLSGGLPGTENSDQARDGTLPYTKDRFYLQ
16 PS I         AEEPTAAAPAEPAPAADEKPEAAVATKEPAKAKPPPRGPKR
18 PS I         AAATETKEAPKGFTPPELDPNTPSPIFAGSTGGLLRKAQVE
CF1 ϵ        (M)TLNLCVLTPNRSIWNSEVKEIILSTNSGQIGVLPNHAPTAT
```

Table V. Quantification of homologies of CF_0II and CF_0I N-terminal sequences with corresponding sequences as aligned in Table IV a. Numbers of identical residues are given. In brackets: Numbers of identities plus conservative and compensating replacements; the groups of residues, which were considered according to Dayhoff [56], are: (M,I,L,V,F), (F,Y,W), (H,R,K), (N,D,E,Q) and (S,T,A,G).

	CF_0II	Syn b′	Ana b′	E.c. b	PS 3 b	CF_0I	Syn b	Ana b
CF_0II	32	13	13	5	7	5	5	4
	(32)	(24)	(21)	(15)	(13)	(13)	(14)	(14)
CF_0I	5	5	7	6	4	34	15	16
	(13)	(12)	(13)	(14)	(14)	(34)	(30)	(29)

Polypeptide CF_0II exists as a polypeptide entity in isolated CF_0CF_1. It is co-isolated in different procedures and co-precipitated together with the other subunits. In properties on SDS gels, immunochemical characteristics and N-terminal amino acid sequence it is distinct from other thylakoid polypeptides in spinach, as far as they are characterized biochemically. It still could be a contamination. There is a wealth of polypeptides in thylakoids with apparent molecular weights between 16 and 20 kDa; after twodimensional separation on gels 21 different polypeptides are listed in this region [32].

The N-terminal sequence of the ferredoxin-NADP reductase binding protein, as determined in this paper, is also new and distinct, *i.e.* different from other thylakoid polypeptides (Table IV b). The supposition of Vallejos *et al.* [33] that the reductase-binding protein is identical to the 16 kDa peripheral protein of the oxygen-evolving complex of PS II is unreasonable, and will be dealed with in a separate publication (R. J. Berzborn and St. Schünemann, in prep.).

Polypeptide CF_0II exists as a biochemical entity besides in spinach also in chloroplasts of several other plants; it is either isolated with the CF_0CF_1 complex, or our specific antibodies against CF_0II cross react and decorate a band on Western blots of thylakoids of these species: *Vicia faba, Avena sativa, Lactuca sativa* var. Romaine, *Pisum sativum, Zea mays, Sorghum bicolor, Lemna gibba* and *Chlamydomonas reinhardii*.

It sometimes forms a heterodimer with CF_0I running on Western blots at about 34 kDa, and shows affinity to $CF_1\varepsilon$ (J. Otto, unpubl.). Cross-links of CF_0II with $CF_1\alpha$, $CF_1\beta$ and $CF_1\gamma$ and with CF_0III have been described [34], although there are difficulties with the nomenclature and identification of subunits in this paper. Since polypeptide CF_0II was not found in isolated b_6f complex (Table I) and decreased in thylakoid subfractions with enriched PS II, CF_0II seems to be found only in CF_0CF_1 preparations.

CF_0II is partially membrane embedded, in agreement with the suitability of the N-terminal sequence to build a transmembrane span. Since it is easily digested by trypsin and accessible to antibodies at the matrix side after CF_1 removal, other part of it are exposed; if the homology to the cyanobacterial F_0b', which is very hydrophilic, also holds for the rest of the sequence of CF_0II not yet published, we do not expect a further span.

From the fact that the accessibilities of CF_0II and CF_0I *in situ* to antibodies and trypsin are similar we concluded [23] that CF_0II may function together with CF_0I as a binding subunit for CF_1, connecting the membrane integral moiety of the ATP-synthase with the peripheral catalytic part.

In analogy to the suggested structure of the two copies of *E. coli* F_0b [35] the CF_0 subunits II and I together may form a 4-helix bundle and constitute (part of) the stalk visible on electron microscopic pictures of CF_0CF_1 [36], penetrating into CF_1. We found by absorption experiments that some immunochemical epitopes on CF_0II and on CF_0I are exposed *in situ*, but others shielded by CF_1.

The results described in this paper suggest a further function: Antibodies against polypeptide CF_0II inhibit rebinding of CF_1 to EDTA-treated thylakoids in agreement with the function just suggested, but in addition inhibit PMS-mediated cyclic photophosphorylation, and after CF_1 removal H^+ efflux from EDTA-treated membranes. Polypeptide CF_0II therefore seems to qualify as an es-

sential and functional subunit of the chloroplast ATP-synthase complex and to guide either protons or some conformational movement up into CF_1.

The residue Glu_{21} within the span could even participate in proton conduction within CF_0. Cyanobacterial F_0b' have the polar Gln in this position (Table IVa). It will be of great value to investigate the effects of interchange of cyanobacterial F_0b and F_0b' with *E. coli* F_0b.

Thus subunit CF_0II may participate in the basic mechanism of ATP-synthesis, but it also may participate in the regulation of this fundamental process in chloroplast thylakoids.

Materials and Methods

Preparation of CF_1 from market spinach [37], chlorophyll [38] and protein [39] determination, SDS-PAGE [40], staining with Coomassie brillant blue (Serva G 250) or silver [16], immunization, agglutination, absorption and inhibition by antibodies [41], Western immunoblots [42], decoration with peroxidase conjugated 2nd antibodies and color development due to oxidation of 4-chloro-1-naphthol by H_2O_2 [43] were carried out according to standard techniques. ELISA [44] was done with horseradish peroxidase conjugated goat *anti*-rabbit (IgG, IgM, BioRad) and measuring the oxidation of *o*-phenylene diamine at 450 nm; 10 µl of antigen, about 10–100 µg, were bound in coating buffer (0.2 M Na_2CO_3, 0.2 M $NaHCO_3$, pH 9.8), to each well of the microtiter plates (Falcon 3912, Becton Dickinson) for at least 3 h at 37 °C. 1% gelatine and antisera were diluted in TTBS (0.05% Tween-20, 20 mM Tris-HCl, 150 mM NaCl, pH 7.5).

Preparation of subunit IV from isolated b_6f complex [19] was done by electroelution [45]. Preparation of the 16 kDa polypeptide of oxygen-evolving complex of PS II was done by anion exchange chromatography on DEAE Sepharose CL-6B in the presence of Triton X-100 from dissolved thylakoids, a by-product of the CF_0CF_1 isolation [4].

The photosynthetic ATP-synthase complex CF_0CF_1 was prepared from spinach thylakoids. After isolation of the membranes in STN (400 mM sucrose, 20 mM Tris-NaOH, pH 7.8, 10 mM NaCl) the pellet after osmotic shock (10 mM NaCl) was resuspended in 20 mM Tris-SO_4, pH 7.7, and 0.1 mM $MgCl_2$, to a Chl concentration of 0.6 mg/ml; an equal volume of 0.5% Triton X-100 in 20 mM Tris-SO_4, pH 7.7 and 10 mM $MgCl_2$ was added, and the suspension stirred for 20 min on ice. After 30 min centrifugation at 38,000 × *g*, 4 °C, the supernatant was adjusted to 60 mM $(NH_4)_2SO_4$ and loaded on DEAE Sepharose CL-6B, equilibrated with 0.25% Triton X-100, 20 mM Tris-SO_4, pH 7.7, 5 mM $MgCl_2$ and 60 mM $(NH_4)_2SO_4$. After washing the protein was eluted with 250 mM $(NH_4)_2SO_4$ in the same buffer, and depending on the purpose concentrated on Amicon XM 100 (10- to 20-fold) and the complex further purified in a sucrose gradient (12–40%, 0.2% Triton X-100, 30 mM Tris-succinate, pH 6.5, 0.1% sonicated phospholipids [2], and the fractions containing CF_0CF_1 frozen at −40 °C; or the eluted protein was in the presence of 1% Na cholate precipitated with solid $(NH_4)_2SO_4$ (250 mg/ml), spun down, dissolved in 40 mM tricine-NaOH, pH 7.8, 2 mM EDTA and kept frozen until use; for electroelution of subunits from SDS gels the fractions from the anion exchange column were frozen immediately in the buffer containing 250 mM $(NH_4)_2SO_4$.

Polypeptide CF_0I and CF_0II were electroeluted [45] from preparative SDS gels [46] according to Lugtenberg [40], CF_0II rechromatographed on SDS gels containing 2.5 M urea [28].

For immunization of rabbits 500–1000 µg of CF_0CF_1, and 100–300 µg of homogeneous CF_0II or CF_0I were used for each treatment [41]. The specificities of the antiserum 278 against CF_0II and of serum 313 against CF_0I were tested, in addition to Western blots with thylakoids, in ELISA with CF_1, CF_0CF_1, and electroeluted polypeptides CF_0I, CF_0II, $CF_1\varepsilon$; within the limits of sensitivity the sera were monospecific. Sometimes polypeptide CF_0II tended to migrate partially at 16 kDa, but partially at 18 kDa without urea on the gels, yielding a double band with serum 278.

Spinach thylakoids for activity measurements were prepared from growth chamber plants as described [47]. During resolution of CF_1 by EDTA from the thylakoid membrane the pH was kept above 7.2 [47]. PMS-mediated cyclic photophosphorylation [47] and inhibition by antibodies [46] was done as described; removal of CF_1 by NaBr treatment [48] monitored by ELISA.

N-terminal sequencing was done in the gas phase by automated Edman degradation (Applied Biosystems Sequenator) and online analysis of the PTH derivatives [49].

Chemicals were of the highest purity available.

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