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Dimeric H^+-ATP synthase in the chloroplast of *Chlamydomonas reinhardtii*

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Abstract

H^+-ATP synthase is the dominant ATP production site in mitochondria and chloroplasts. So far, dimerization of ATP synthase has been observed only in mitochondria by biochemical and electron microscopic investigations. Although the physiological relevance remains still enigmatic, dimerization was proposed to be a unique feature of the mitochondrion [Biochim. Biophys. Acta 1555 (2002) 154]. It is hard to imagine, however, that closely related protein complexes of mitochondria and chloroplast should show such severe differences in structural organization. We present the first evidences for dimerization of chloroplast ATP synthases within the thylakoid membrane.

By investigation of the thylakoid membrane of *Chlamydomonas reinhardtii* by blue-native polyacrylamide gel electrophoresis, dimerization of the chloroplast ATP synthase was detected. Chloroplast ATP synthase dimer dissociates into monomers upon incubation with vanadate or phosphate but not by incubation with molybdate, while the mitochondrial dimer is not affected by the incubation. This suggests a distinct dimerization mechanism for mitochondrial and chloroplast ATP synthase. Since vanadate and phosphate bind to the active sites, contact sites located on the hydrophilic CF_1 part are suggested for the chloroplast ATP synthase dimer. As the degree of dimerization varies with phosphate concentration, dimerization might be a response to low phosphate concentrations.

Keywords: Chloroplast ATP synthase; Blue-native electrophoresis; *Chlamydomonas reinhardtii*; Membrane protein complex; Thylakoid membrane

1. Introduction

The H^+-ATP synthase located in unstacked regions of the thylakoid membrane has a central role in ATP production by the chloroplast. While dimeric or oligomeric association of ATP synthase complexes in mitochondrial inner membrane was demonstrated by electron microscopy [1,2] and biochemical investigations [3,4], chloroplast ATP synthase was suggested to exist merely as a monomer [5]. The ATP synthase dimer was proposed to be a unique feature of mitochondria [5]. Functions like protein complex stabilization [3], control of enzymatic activity [6,7] and the formation of tubular cristae inside the mitochondria [8,9] have been assigned to the dimerization of ATP synthase. It is, however, surprising that the closely related protein complexes of mitochondria and chloroplast should show such pronounced differences in structural organization.

Examining the proteome of the inner mitochondrial membrane of *Chlamydomonas reinhardtii*, the mitochondrial ATP synthases were demonstrated to be organized as extraordinarily stable dimers, but no dimer of the chloroplast ATP synthase was observed [10].

Studies with protein cross-linking agents in yeast showed that subunit 4 and two dimer specific subunits *e* and *g* are involved in dimerization [2]. Besides subunit 4 of *Saccharomyces cerevisiae*, which is homologous to subunit b in mammalian mitochondria and to subunits I and II of chloroplast ATP synthase, none of these subunits *e* and *g* is known to have a counterpart within the chloroplast.

Blue-native polyacrylamide gel electrophoresis (BN-PAGE) is a marvelous analytical tool as has been demonstrated for the investigation of protein complexes of the inner mitochondrial membrane [11,12]. The separation of membrane protein complexes and supercomplexes in structurally intact form including the isolation of dimeric ATP

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synthase in yeast [3], mammalian [4], algae [10] and higher plant mitochondria [13] could be achieved. In addition, BN-PAGE allowed the preparative isolation of structural and functional intact chloroplast ATP synthase, as well as the separation of the soluble CF_1 and the membrane integral subcomplex CF_o of spinach [14,15]. These protein complexes isolated by BN-PAGE were used for 2-D crystallization and helped to establish the stoichiometry of 14 protomers III in the proton transducting rotor [16,17]. The first analytical use of BN-PAGE in combination with SDS-PAGE for the investigation of the thylakoid membrane of spinach chloroplasts visualized the protein subunit composition of all protein complexes involved in photophosphorylation in a single 2-D gel [18]. Recently, BN-PAGE served as a tool for differential display of thylakoid membrane proteins of *C. reinhardtii* grown at different culture conditions [19]. The association of photosystem I (PSI) and light-harvesting complex I (LHCI) was shown to be diminished in cultures grown photomixotrophically as compared to algae grown under photoautotrophic conditions. No indication for a chloroplast ATP synthase dimer was found at that time, despite the application of various solubilization conditions, while a mitochondrial dimer was present.

For further improvement of thylakoid isolation and solubilization, two strategies were employed, the inhibition of kinases and phosphatases and the reduction of force applied during cell disruption. Protein phosphorylation has a great impact on protein complexes, as well as on the overall structure of the thylakoid membrane [20]. Enzymatic activity and structural integrity of membrane protein complexes like the chloroplast ATP synthase are sensible against mechanical stress [21]. Upon reducing the pressure for disruption of cells in the French press, we could observe the dimer of the chloroplast ATP synthase. Estimation of the apparent mass of the protein complex and analysis of proteins by MALDI-TOF-MS led to an unambiguous identification of the chloroplast ATP synthase dimer. Since the formation of a dimer was thought to be an exclusive feature of the mitochondrial ATP synthase [5], this result is rather unexpected.

In this report biochemical evidence for the dimeric association of the chloroplast ATP synthase is presented for the first time and clue is given for the location of the interacting sites, involved in dimer formation, at the CF_1 part of the ATP synthase. In addition, the exceptional stability [10] of the mitochondrial ATP synthase dimer is confirmed.

2. Material and methods

2.1. Culture conditions

C. reinhardtii strain 83−81 (*cw15*-mutant) (Sammlung für Algenkulturen, Göttingen, Germany) was grown photomixotrophically as described [19] using Tris-acetate-phosphate medium [22]. Cultures were harvested by centrifugation (3500 × *g*, 4 °C, 10 min) at the beginning of the final illumination period after 84 h growth. The cell pellet was resuspended in either of two different media at 4 °C. The first contained 1 mM $MnCl_2$, 5 mM $MgCl_2$, 250 mM sorbitol, 35 mM HEPES (pH 7.8), and 2 mM Na_2EDTA; the second contained in addition 5 mM NaF and 5 mM Na_3VO_4. Cells were broken in a pre-cooled French pressure cell at 90 bar. After centrifugation (3000 × *g*, 4 °C, 3 min), homogenization of the pellet in 5 mM $MgCl_2$ solution or 5 mM $MgCl_2$, 5 mM NaF, and 5 mM Na_3VO_4, respectively, disrupted intact chloroplasts.

The suspension of the broken chloroplasts was centrifuged (3000 × *g*, 4 °C, 3 min). The pellet of the thylakoid membranes was supplemented with 10% (w/v) glycerol, frozen in liquid nitrogen, and stored at −20 °C.

2.2. Solubilization of the thylakoid membranes

Solubilization of thylakoid membranes was performed at 4 °C for 30 min at a chlorophyll concentration of 0.25 mg/ml in a medium containing 200 mM ε-aminocaproic acid (6-aminohexanoic acid), 5 mM $MgCl_2$, 5 mM $MnCl_2$, 10% (w/v) glycerol, 20 mM tricine, 5 mM DTT, and 1% digitonin (Calbiochem, high purity, water-soluble, cat. no. 300410) in the dark under continuous agitation. Upon solubilization, insoluble material was removed by centrifugation (20,000 × *g*, 4 °C, 1 h). The supernatant was loaded directly onto blue-native gradient gels.

In control experiments, NaF, Na_3VO_4, Na_3PO_4, Na_2MoO_4, or a mixture of NaF and Na_3VO_4 was added during solubilization, but not in the previous homogenization step.

2.3. 2-D Blue-native/Tricine-SDS-PAGE and colorless-native PAGE

Blue-native PAGE was performed using the Hoefer SE 600 system (18 × 16 × 0.15 cm^3, 10 lanes) as described previously [11,14] with slight modifications [19]. The gels contained 200 mM ε-aminocaproic acid and 50 mM Bis-Tris. Stacking gels had a total acrylamide concentration of 3% and separating gels acrylamide gradients from 3.5 to 20%.

One-hundred micrograms of solubilized protein was applied per lane, as determined by the Lowry−Hartree method [23] after protein precipitation with deoxycholic and trichloroacetic acid.

Colorless-native PAGE (CN-PAGE) was performed under the same conditions as the BN-PAGE omitting the Coomassie G-250 from the cathode buffer with gels supplemented with 0.02% (w/v) digitonin. BN-PAGE sample buffer [11] was added to one lane of the colorless-native gel to visualize the running front.

After electrophoresis, gels were stained with Coomassie R-250 or lanes of the BN-gel were cut out and incubated in

a solution of 1% (w/v) SDS and 1% (w/v) β-mercaptoethanol at 20 °C for 30 min. Subsequently, lanes were analyzed by Tricine-SDS-PAGE in second dimension on a gel with two stacking gels, one native and one denaturing, with a total acrylamide concentration of 10% and a separating gel with 16.5% [24].

The ATPase activity of the mitochondrial ATP synthase was monitored in blue-native and colorless-native gels according to Ref. [25] with slight modifications. The gel was incubated over night in a solution of 75 mM Tris, pH 7.8, 5 mM magnesium acetate, 2 mM lead acetate and 5 mM ATP. To measure the ATPase activity of the chloroplast ATP synthase, 25% (v/v) methanol has to be added to the assay, as hydrolyzation reaction is inhibited in the native enzyme.

2.4. Protein identification

After electrophoresis, gels were silver-stained [26,27]. Protein bands of interest were excised inside a laminar flow hood. Silver stained spots were destained [28], and in-gel digestion was performed with trypsin [19]. After extraction of the peptides with 50% (v/v) acetonitrile/0.5% (v/v) trifluoroacetic acid, the solvent was removed completely by lyophilization. Lyophilized samples were dissolved in 0.5% (v/v) trifluoroacetic acid by sonification for 3 min and desalted using μ-C18 ZipTips (Millipore). Elution was performed directly onto stainless steel target plate with 2.5 mg/ml 4-hydroxycinnamic acid in 50% (v/v) acetonitrile/0.1% (v/v) trifluoroacetic acid.

Peptide samples were analyzed with a Voyager-DE PRO (Applied Biosystems) MALDI time-of-flight mass spectrometer operating in positive reflection mode. Spectra were acquired in a mass window from 800 to 3800 *m/z*, applying an automatic external five-point calibration. For subsequent internal mass calibration two trypsin autoproteolysis peaks were used. The ‘‘Mascot’’ software www.matrixscience.com) was employed to match detected masses against the NCBInr database. The search included one possible missing cleavage site and the possible oxidation of methionine.

3. Results

The green algae *C. reinhardtii* was grown under photomixotrophic conditions, i.e., using both light and acetate as energy source. After disruption of the algae in a French press at reduced pressure as compared to Ref. [19] and lysis of the chloroplasts by osmotic shock, thylakoids were sedimented by centrifugation. One part of the cultures was prepared in the presence of the phosphatase inhibitors Na_3VO_4 and NaF, while the other was not treated with inhibitors. The digitonin solubilized thylakoid membranes of both preparations were analyzed by BN-PAGE. After additional staining with Coomassie R-250, about 16 bands corresponding to different protein complexes and supercomplexes of the photophosphorylation chain could be visualized (Fig. 1, lanes A and B). The identity of these complexes and supercomplexes was determined unambiguously by denaturing SDS-PAGE in second dimension and subsequent peptide mass fingerprinting of selected protein spots (Fig. 2, Table 1).

To estimate the apparent mass of the protein complexes in the blue-native gel, a high molecular mass standard (HMW) and mildly digitonin-solubilized bovine heart mitochondria (Fig. 1, lane BHM) served as reference [4]. Protein complexes from mildly solubilized bovine heart mitochondria are well characterized and are often used to estimate the mass of complexes in blue-native gels [10,29].

In addition to protein complexes described in earlier investigations [18,19], a band corresponding to the dimer of the chloroplast ATP synthase $(CF_oF_1)_2$ is observed with an apparent mass of approximately 1300 kDa (Fig. 1). The band of dimeric chloroplast ATP synthase $(CF_oF_1)_2$ is significantly more pronounced in the absence of phosphorylase inhibitors Na_3VO_4 and NaF (Fig. 1, lane B) as in the presence (Fig. 1, lane A). The intensity of the monomeric chloroplast ATP synthase, which is displayed at about 600

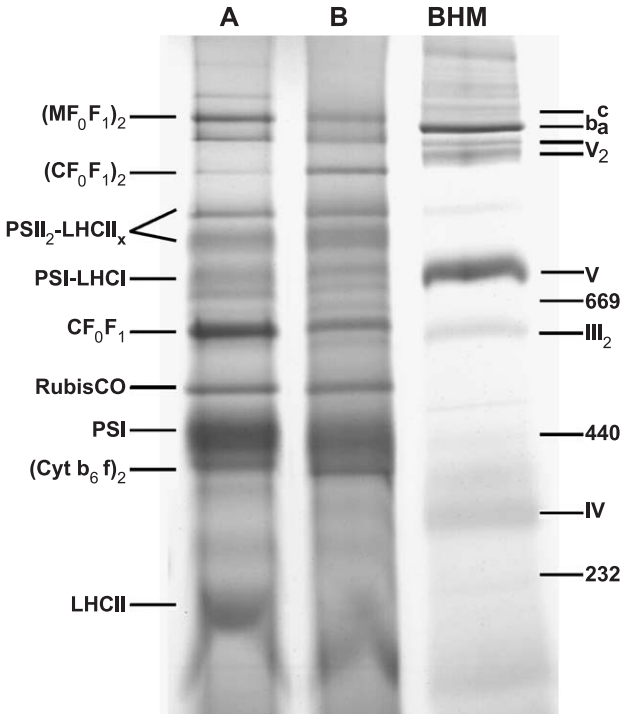

Fig. 1. Solubilized thylakoid membranes isolated in the presence of phosphatase inhibitors Na_3VO_4 and NaF during isolation (A), or in the absence of phosphatase inhibitors (B) and mildly solubilized bovine heart mitochondria (BHM), separated by BN-PAGE, 3–20% gradient gel after additional staining with Coomassie R-250. Supercomplexes from mildly solubilized bovine heart mitochondria are well characterized [4] and can be used to estimate the mass of protein complexes in blue-native gels [10,28]. Positions of HMW marker proteins are indicated with the corresponding molecular mass. c: $I_1III_2IV_2$, 1900 kDa; b: $I_1III_2IV_1$, 1700 kDa; a: I_1III_2, 1550 kDa; V_2: mitochondrial ATP synthase dimer, 1450 kDa; I: NADH dehydrogenase, 1000 kDa; V: mitochondrial ATP synthase monomer, 750 kDa; III_2: cytochrome *c* reductase dimer, 600 kDa; IV: cytochrome *c* oxidase, 230 kDa.

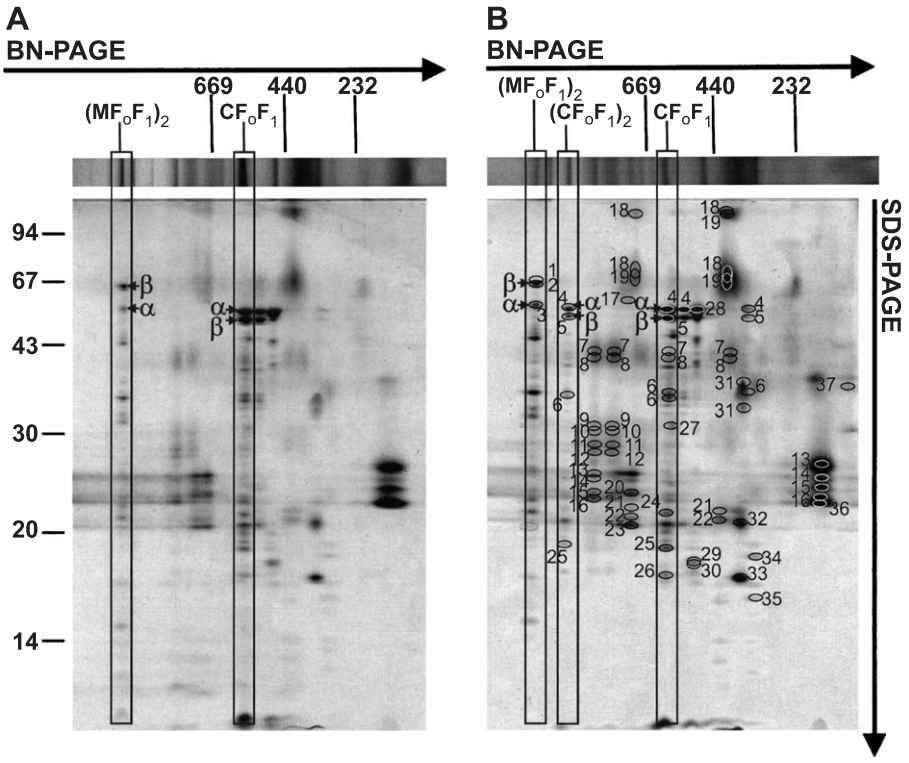

Fig. 2. 2D-BN/SDS-PAGE of solubilized thylakoid membranes incubated with phosphatase inhibitors Na_3VO_4 and NaF during isolation (A), as well as of solubilized thylakoid membranes without phosphatase inhibitors (B). The boxes highlight the vertical areas where bands of the ATP synthase complex dimer from mitochondria as well as monomer and dimer from chloroplast ATP synthase are observed. The beta subunits of mitochondrial and chloroplast ATP synthase, which differ significantly in the migration behavior in the gel, are marked in the gels, as well as the alpha subunit. Protein spots identified by MALDI-MS have been labelled with numbers corresponding to Table 1.

kDa, is increased in lane A, while the intensity of the band of the dimer $(CF_oF_1)_2$ is reduced (Fig. 1, lane A). If the intensity of the chloroplast ATP synthase dimer band is normalized to the sum of the intensities of chloroplast monomer and dimer bands, the relative proportion of the chloroplast ATP synthase dimer is estimated to be 44% in lane B. In lane A, in response to the presence of the two inhibitors during cell breakage and isolation of the thylakoid membrane, the proportion of the dimer drops to 9% only.

To exclude any Coomassie-induced artificial aggregation of the chloroplast ATP synthase, colorless-native PAGE (CN-PAGE) was applied to analyze solubilized thylakoid membranes of both preparations (Fig. 3). The CN-PAGE technique omits the dye Coomassie blue G-250, which is used to transfer negative charge to the protein complexes under BN-PAGE conditions. Therefore, separation by CN-PAGE is based on the native charge of the protein complexes. CN-PAGE is reported to be more gentle than the BN-PAGE and to preserve the native structure of the protein complexes [30,31]. While the resolution of the CN-PAGE is inferior, the results correspond to the results obtained by the blue-native technique. In the absence of the phosphorylase inhibitors (Fig. 3, lane B) a pronounced band of the chloroplast ATP synthase dimer is observed, while it is a very weak band in lane A. Upon addition of Coomassie G-250 to the sample buffer the separation is equivalent to the separation in a blue-native gel. The resolution of the electrophoretic separation increases after the addition of the Coomassie dye (Fig. 3, lane BN-A). The content of dimeric chloroplast ATP synthase is not increased, as shown by applying identical, vanadate treated samples to lane A and BN-A, supplementing the sample in lane BN-A with Coomassie G-250.

As the number of mitochondria is increased due to the presence of acetate as additional carbon source during photomixotrophic growth of the algae, and since the thylakoids were isolated only by differential centrifugation, some mitochondrial proteins, in particular the dimer of the mitochondrial ATP synthase, appear in the gels. However, the mitochondrial dimer is clearly separated at higher molecular mass from the corresponding chloroplast dimer. It can serve as internal standard to compare the properties of the mitochondrial and chloroplast ATP synthase dimer directly in a single sample. As recently reported [10], the dimer of mitochondrial ATP synthase of *C. reinhardtii* with an apparent mass of about 1750 kDa is indeed significantly larger and more stable than the dimer from bovine heart mitochondria (Fig. 1, lane BHM, V_2, 1450 kDa). Despite the variety of solubilization conditions employed, under no condition a monomer of the mitochondrial ATP synthase of *C. reinhardtii* could be detected in any gel. The ATPase activity of the mitochondrial ATP synthase dimer can be

Table 1

No.[a]	Protein	NCBInr[b]	Score[c]
1	P60, MF_oF_1	29786351	135
2	Beta subunit, MF_oF_1	282721	146
3	Alpha subunit, MF_oF_1	1495363	220
4	Alpha subunit, CF_oF_1	28269773	247
5	Beta subunit, CF_oF_1	336667	166
6	Gamma subunit, CF_oF_1	67881	116
7	CP47, PSII	7443181	119
8	CP43, PSII	131278	68
9	D2, PSII	28269779	97
10	D1, PSII	28269786	91
11	CP29, PSII	15430560	151
12	CP26, PSII	12060444	80
13	LhcII-1.3[d]	15430554	72
14	LhcII-1.1[d]	99383	67
15	LhcII-4[d]	15430566	70
16	LhcII-3[d]	15430564	73
17	Chaperonin 60, chloroplast	2129459	88
18	PSI-A, PSI	17380187	92
19	PSI-B, PSI	26051282	76
20	LHCI-7[d]	27542569	67
21	PSI-D, PSI	1644293	67
22	PSI-F, PSI	4689368	65
23	LHCI-6[d]	421766	68
24	Delta subunit, CF_oF_1	1134976	60
25	Subunit I, CF_oF_1	32699336	160
26	Epsilon subunit, CF_oF_1	114595	62
27	Cytochrome c_1, complex III	27525859	65
28	Large subunit, RubisCO	68154	191
29	Small subunit 1, RubisCO	132076	74
30	Small subunit 2, RubisCO	132092	77
31	Cytochrome *f*, cytochrome b_6f	475066	123
32	Rieske protein, cytochrome b_6f	541541	104
33	Subunit 4, cytochrome b_6f	129855	60
34	Subunit II, complex IV	13604153	55
35	Histone H4	1708110	77
36	Peroxiredoxin	11995220	67
37	Subunit A, GAPDH	1730149	193

Protein assignments were performed using the publicly available "Mascot" software against NCBInr database. Taxonomy was limited to green plants excluding *Arabidopsis thaliana* and *Oryza sativa*.

[a] Numbers representing labels in Fig. 2.

[b] NCBInr database accession number.

[c] Scores obtained by "Mascot". Scores of 60 and above are considered as significant.

[d] Names assigned to LHCI and LHCII protein subunits according to Tokutsu et al.[44] and Teramoto et al.[45], respectively.

monitored directly in the gel. However, such a test cannot be performed with the intact chloroplast dimer, as the subunit epsilon, which inhibits the ATP hydrolysis reaction, has to dissociated from the complex by the addition of 25% (v/v) methanol in order to display the ATPase activity of the chloroplast ATP synthase.

The absolute content of mitochondrial ATP synthase dimers differs in lanes A and B of Fig. 1, as the amount of mitochondrial contaminations in the thylakoid samples is not constant in different preparations. In addition, the overall amount of protein applied to lane A seems slightly higher than in lane B, although the amount of sample applied to the lanes was adjusted by the chlorophyll concentration. However, Fig. 5A shows that the intensity of the mitochondrial ATP synthase dimer band is not correlated to the presence or concentration of additional phosphate, vanadate or molybdate ions, as the applied thylakoid samples originate from the same preparation.

To elucidate the origin of dissociation of the chloroplast ATP synthase dimer a thylakoid membrane sample, which was isolated from the green algae in the absence of the inhibitors Na_3VO_4 and NaF and therefore exhibited a dimer band, was supplemented with 5 mM NaF, 5 mM Na_3VO_4, or both 5 mM NaF and 5 mM Na_3VO_4, during solubilization and subsequently analyzed by BN-PAGE. A densitometric analysis (Fig. 4) shows clearly that the dissociation of the dimer is merely dependent on Na_3VO_4. The samples solubilized in the presence of Na_3VO_4 or a mixture of Na_3VO_4 and NaF displays only a weak dimer but a more intense monomer band. The ratio between staining intensities of dimeric and the sum of dimeric and monomeric chloroplast ATP synthase drops significantly in the presence of Na_3VO_4 (Fig. 4B). The staining ratio in the presence of NaF is slightly but not significantly higher as compared to the untreated sample. For further analysis of the effect of different ions on the integrity of the chloroplast ATP synthase dimer, the experiment was repeated with increasing concentrations of Na_3PO_4, Na_3VO_4, and Na_2MoO_4 in the solubilization media (Fig. 5). While the dimer is dissociated to the monomer by phosphate and vanadate, the dimer is

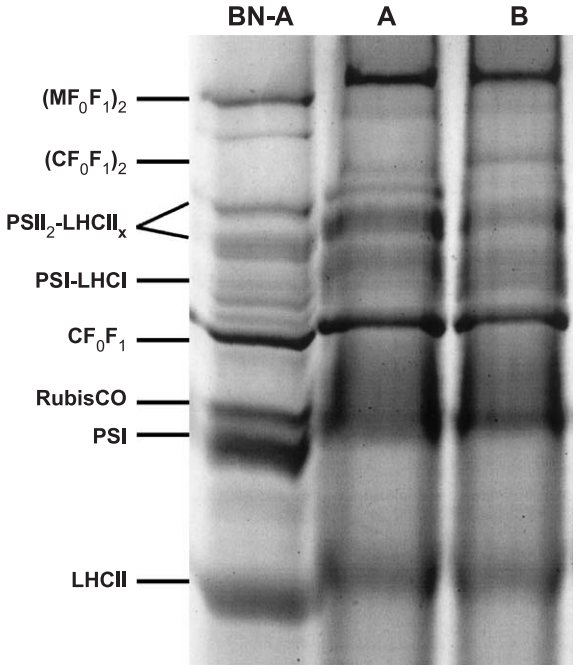

Fig. 3. Solubilized thylakoid membranes isolated in the presence of phosphatase inhibitors Na_3VO_4 and NaF during isolation (A) or in the absence of phosphatase inhibitors (B), separated by CN-PAGE, 3–20% gradient gel after additional staining with Coomassie R-250. The sample applied to lane BN-A is identical to the sample applied to lane A, but was supplemented with Coomassie G-250 prior to the electrophoresis. The separation observed in this lane is equivalent to the separation characteristics of a blue-native gel.

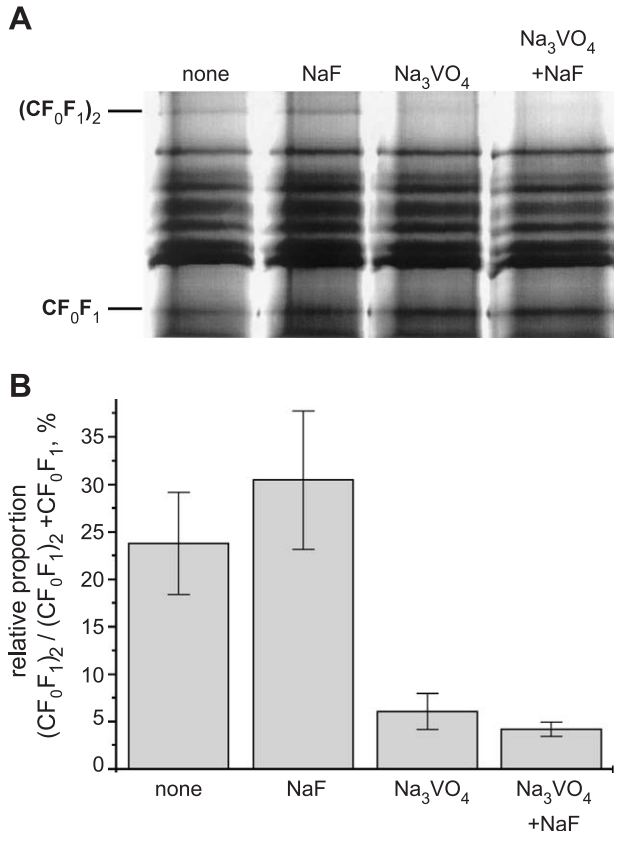

Fig. 4. Densitometric analysis of chloroplast ATP synthase dimers after separation by BN-PAGE. (A) Solubilized thylakoid membranes untreated or treated with NaF, with Na_3VO_4, or with both NaF and Na_3VO_4 were separated on a blue-native gradient gel (3.5%–16%) and stained with Coomassie R-250. (B) The intensity of the band of the chloroplast ATP synthase dimer of each lane was normalized to the sum of the intensities of dimer and monomer band. Error bars symbolize deviations caused by different modes of background correction. Lanes containing Na_3VO_4-treated thylakoid membranes show only very weak dimer bands.

quite stable against elevated concentration of molybdate. By densitometric analysis and normalization to the sum of ATP synthase monomer and dimer band intensity (Fig. 5B), it can be observed that the effect of vanadate on the stability of the chloroplast ATP synthase dimer is slightly more pronounced than the effect of phosphate, while even 5 mM molybdate shows no noticeable effect on the dimer.

To characterize the protein complexes observed in Fig. 1, corresponding lanes were cut out and placed on top of a denaturing SDS-PAGE. During SDS-PAGE in second dimension, the complexes are separated into their protein subunits. Since protein complexes migrate in their native state in the first dimension, their subunit pattern appears on vertical lines in the 2-D gel.

In the 2-D gels (Fig. 2A and B) all subunits of membrane protein complexes involved in photophosphorylation are present. The chloroplast ATP synthase dimer $(CF_oF_1)_2$ and monomer CF_oF_1, as well as the mitochondrial ATP synthase dimer $(MF_oF_1)_2$ have been marked in the gels. Monomer and dimer of the chloroplast ATP synthase show the same characteristic subunit pattern. In addition to the ATP synthase complexes in Fig. 2 A and B, dimeric photosystem II associated with light-harvesting complex II ($PSII_2$-$LHCII_x$), photosystem I associated with light-harvesting complex I (PSI-LHCI), RubisCO, monomeric PSII, monomeric PSI, and cytochrome b_6f were identified in the gels by peptide mass fingerprinting as described in [19]. Proteins identified are marked in Fig. 2 and summarized in Table 1. The ATP synthase subunits from mitochondria can be clearly distinguished from those of the corresponding chloroplast proteins based on their subunit pattern. The beta subunit of

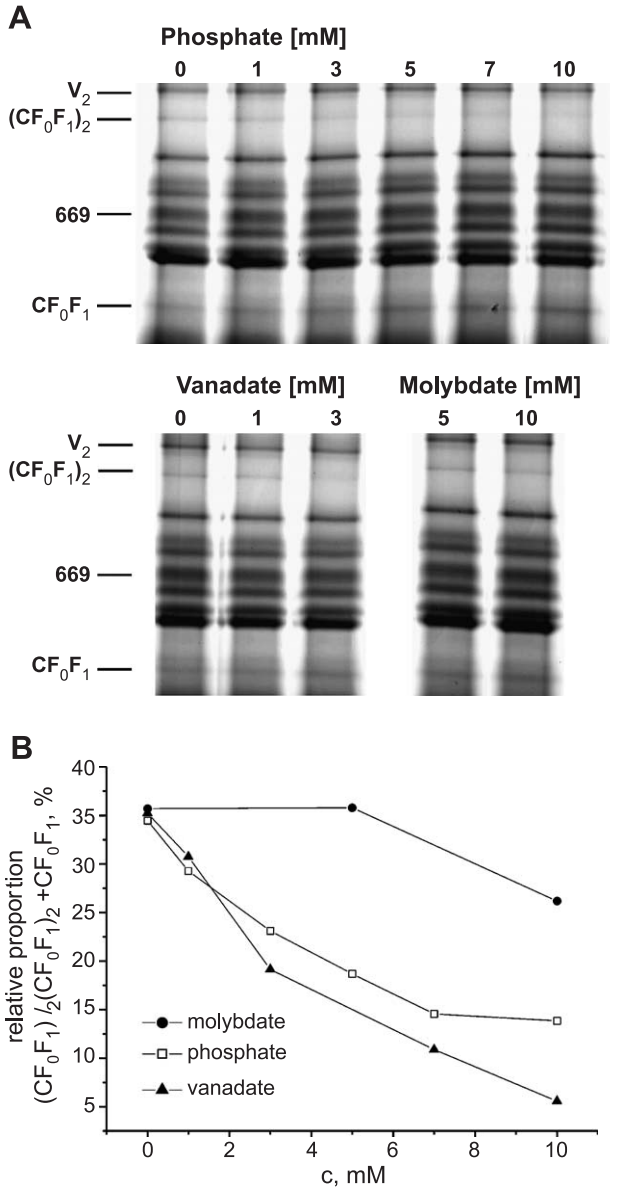

Fig. 5. (A) Effect of phosphate, vanadate, or molybdate present during solubilization on chloroplast ATP synthase dimers. Ions and their concentrations applied during solubilization are indicated. (B) Densitometric analysis of solubilized thylakoid membranes separated by BN-PAGE, 3.5–16% gradient gel, after additional staining with Coomassie R-250. The intensity of the dimer band was normalized to the sum of the intensity of dimer and monomer band of the chloroplast ATP synthase.

mitochondrial ATP synthase from *C. reinhardtii* has a significantly higher mass than the corresponding subunit in chloroplast or bovine mitochondria and it is even higher than the mass of the mitochondrial alpha subunit, which is a feature that can be clearly recognized in the gels (Fig. 2A and B).

To examine the identity of the protein subunits, protein spots were cut out of the gel, trypsinated, and analyzed by MALDI-TOF-MS. Subunits alpha and beta as well as the ATP synthase associated protein P60 [10] could be identified for the dimer of the mitochondrial ATP synthase. For all chloroplast ATP synthase complexes the subunits alpha, beta, gamma and subunit I could be unambiguously identified. Results for subunit delta and subunit epsilon were only significant for the more abundant chloroplast ATP synthase monomer. The protein spots of subunit IV, subunit III and the oligomer III_{14} of the chloroplast ATP synthase cannot be identified by peptide mass fingerprinting, as the number of tryptic peptides within the mass range appropriate for MALDI-MS analysis is not sufficient for unambiguous identification. A low abundant $PSII_2$ species associated with LHCII and the dimer of the mitochondrial complex III show an almost identical migration behavior in the first dimension. The centers of proteins spots originating from these protein complexes tend to be slightly shifted (complex III, Fig. 2B, spot 27) or to be significantly weaker (PSII, Fig. 2B, spots 7 and 8). The protein subunits can be distinguished by peptide mass fingerprinting after SDS-PAGE in the second dimension.

Since corresponding subunits of mitochondrial and chloroplast ATP synthase subunits show only homologies of about 60% based on their amino acid sequence, mass spectrometric analysis can clearly differentiate between the mitochondrial or chloroplast homologs of the respective proteins. The mass spectra measured of the tryptic peptides from the mitochondrial and chloroplast ATP subunits show no significant similarities and subsequent identification of the proteins in databases is unambiguous.

4. Discussion

By BN-PAGE and subsequent identification by MALDI-MS analysis, the existence of dimers of chloroplast H^+-ATP synthase could be demonstrated for the first time. Recently, the dimerization was proposed to be a special feature of the mitochondrial ATP synthase (complex V) only [5].

BN-PAGE is a technique widely employed to characterize subunit composition and oligomeric state of protein complexes. In addition, the technique gives information about the molecular mass of protein complexes. Investigations comparing size exclusion chromatography, sucrose-gradient centrifugation, and BN-PAGE to elucidate molecular mass or dimerization of protein complexes show clearly that all three methods lead to comparable results, although molecular masses obtained by the different methods differ by up to 25% [32,33]. By comparison with analytical ultracentrifugation, it was demonstrated that the oligomeric state of membrane transport proteins is reflected reliably by blue-native gel electrophoresis [34]. Analyzing the cytochrome b_6f dimer, mass derived from the amino acid sequence of 230 kDa by BN-PAGE and size-exclusion chromatography led to molecular masses of 250 and 190 kDa, respectively, whereas single particle electron microscopy led to an estimated mass below 250 kDa [35].

Masses obtained by BN-PAGE are modulated by protein structure and differences in Coomassie binding [34]. However, the oligomeric character of proteins and protein complexes is preserved and unambiguously identified. Protein-dependent characteristics like hydrophobicity and binding capacity for the dye Coomassie have largely the same magnitude for monomeric and oligomeric structures. Thus, separation of monomers and dimers is mainly based on size.

All protein complexes as well as protein supercomplexes observed in the gels (Figs. 1 and 2) have been described in the literature, but not the dimer of the chloroplast ATP synthase. Mitochondrial ATP synthase dimers have been found in mammalian and fungal mitochondria [4,31]. Recently, dimers have been observed in the mitochondria of *C. reinhardtii* [10]. As no monomers of the mitochondrial ATP synthase, but exclusively dimers are present in the gels (Figs. 1 and 2), the mitochondrial dimer seems to be very stable [10]. Analysis by CN-PAGE excludes any Coomassie-induced aggregation. The content of dimeric and monomeric chloroplast ATP synthase is independent from presence of the Coomassie G-250 during the electrophoretic separation. The Coomassie dye, which is used to transfer anionic charge to the protein complexes under BN-PAGE conditions, only increases the resolution of the separation method.

Since no protein precipitation and no unusual protein associations occur in the gels, we conclude that the dimerization of the chloroplast ATP synthase is not caused by unspecific association during the isolation or solubilization process, but reflects the native state in the thylakoid. In two neighboring lanes of the same gel, one sample displays a dimer of the chloroplast ATP synthase, while the other incubated with the phosphatase inhibitor Na_3VO_4 during cell disruption (Fig. 1) or during solubilization of the thylakoid membrane (Figs. 4 and 5) shows only a faint dimer band but a more intense monomer band. This excludes any artifacts due to electrophoretic separation and demonstrates a specific effect.

While the employment of Na_3VO_4 may have a significant effect on the ionic strength of the cell disruption media, the influence on the ionic strength during solubilization can be neglected, as 200 mM ε-aminocaproic acid has a higher impact on the ionic strength in the solubilization media than 5 mM Na_3VO_4, and even 10 mM Na_2MoO_4 does not show a comparable effect on the chloroplast ATP synthase dimer (Fig. 5). Incubation with phosphate or the phosphatase inhibitor vanadate, but not with NaF, leads to the dissoci-

ation of the dimer of the chloroplast ATP synthase into monomers.

The content of mitochondrial ATP synthase dimer is mainly influenced by the varying content of contamination by mitochondria in the thylakoid samples (Fig. 1). The variation is less pronounced in Fig. 4, as the thylakoid samples solubilized in the presence of dissociating agents vanadate or phosphate originate from the same thylakoid preparation and variations are only due to slightly different solubilization conditions. There is no correlation between the amount of mitochondrial ATP synthase dimer and the presence, concentration, and kind of dissociating agent.

As the mitochondrial ATP synthase dimer is not significantly influenced by the incubation, a different mechanism of dimer formation or different dimer contact sites may be present in dimers of mitochondrial and chloroplast ATP synthase. The formation of mitochondrial ATP synthase dimers is associated with dimer specific subunits such as subunits *e* and *g* in mitochondria of *S. cerevisiae* within the membrane integral F_o subcomplex [3]. A high protein background originating from the high abundant light-harvesting complex subunits makes it difficult to identify such additional ATP synthase subunits between 35 and 15 kDa specific for the dimeric complex in our gels by visual inspection (Fig. 2B) as well as by peptide mass fingerprinting. Therefore, the existence of such subunits in the chloroplast cannot be excluded. An artificial dimerization of the chloroplast ATP synthase by binding proteins released from the mitochondrion during preparation of the thylakoid membrane is unlikely, since a hypothetical association of these proteins should not depend on the presence or absence of phosphate or vanadate, respectively.

Furthermore, if formation of chloroplast ATP synthase dimers would be triggered by protein phosphorylation or dephosphorylation of ATP synthase subunits, incubation with NaF might have an effect on the intensity of the dimer band. Inhibition of both kinases and phosphatases directly during cell disruption and during thylakoid membrane preparation should arrest the current protein phosphorylation. In the presence of kinases and ATP, protein-phosphorylation should be increased by the inhibition of phosphatases. Since no significant amount of ATP is available, inhibition of phosphatases during solubilization should not influence the degree of protein phosphorylation. Thus, regulation of chloroplast ATP synthase dimerization by a protein phosphorylation signal is unlikely.

Molybdate and vanadate, as well as phosphate form tetrahedral oxoanions and are used as competitive phosphatase inhibitors. However, molybdate has a lower charge and larger diameter than the corresponding phosphate and vanadate ions. While it is unclear whether the insensitivity of the chloroplast ATP synthase dimer to molybdate is a consequence of the molybdate ion size, it is obvious that the dissociation by phosphate and vanadate (Fig. 5) is not caused by the increased ionic strength or other unspecific effects. As the degree of dimerization is correlated to phosphate concentration of physiological relevance [21] (Fig. 5), dimerization seems to be a response to conditions with low phosphate.

Vanadate is a phosphatase inhibitor resembling the structure of phosphate and binds to the active site of enzymes catalyzing dephosphorylation processes by stabilizing the trigonal bipyramidal geometry of the dephosphorylation transition state, as has been demonstrated by X-ray diffraction for the complex formed with myosin [36]. Since the ATP synthase itself is a phosphatase and vanadate has been shown to bind to the active site of mitochondrial ATP synthase [37], interaction between the highly homologous active site of the chloroplast ATP synthase [38] and vanadate has to be expected. The hydrophilic part of the ATP synthase, the CF_1 part, which contains the active sites for phosphorylation/dephosphorylation, has a much larger diameter than the membrane intrinsic CF_o subcomplex, as is shown by single-particle electron microscopy for the chloroplast ATP synthase from spinach [39] and by the structures available for its subcomplexes [17,38,40]. Binding of ADP/P_i and release of ATP induce conformational changes in the active sites [41,42], as well as in the whole CF_1

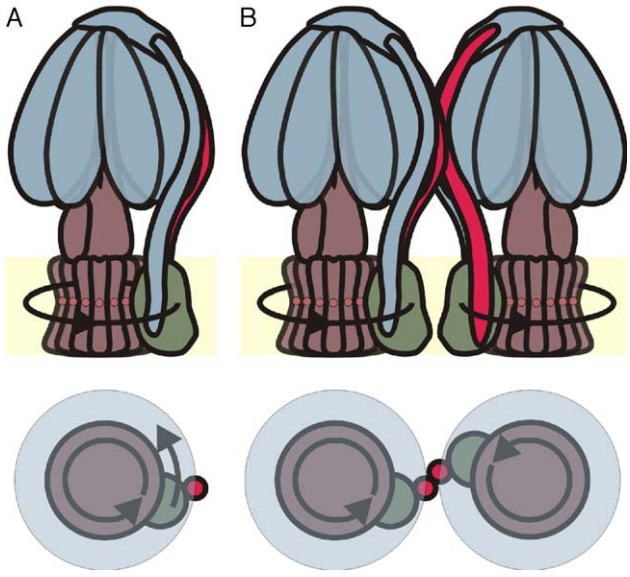

Fig. 6. Hypothetic model of the chloroplast ATP synthase dimerization. The ATP synthase couples vectorial proton translocation across the thylakoid membrane to the rotational movement of the oligomer III_{14}. Subunits γ and ε are associated with the oligomer III_{14} and drive the formation of ATP by inducing conformational changes within the catalytic hexamer $\alpha_3\beta_3$. Subunits γ, ε and III_{14} (brown) form the rotor; subunits $\alpha_3\beta_3$, δ, II (blue), IV (green) and I (red) form the stator. (A) In the monomeric state of the ATP synthase, neither rotor nor stator is rigidly anchored in the lipid bilayer in a manner excluding rotational movements. In addition to conformational changes in $\alpha_3\beta_3$, the movement of the rotor exerts a force on the stator, which results in a slow rotation of the stator. As a consequence, both rotor and stator are rotating unidirectional. (B) In the dimeric state, interactions between ATP synthases prevent unidirectional movement of rotor and stator. This interaction is mediated by the peripheral hydrophilic parts of subunits I (red) associated with the $\alpha_3\beta_3$ hexamer.

subcomplex. Binding of vanadate may induce conformational changes within the complex [37], which might be responsible for the dissociation of the chloroplast ATP synthase dimer. Therefore, in contrast to mitochondrial ATP synthase, where dimerization seems to be driven by the membrane intrinsic F_o subcomplex [2,3,7], interacting sites for dimerization of the chloroplast ATP synthase appear to be located in the hydrophilic part. As the subunit I is the only subunit of the chloroplast ATP synthase showing homology to mitochondrial subunits involved in the dimerization of mitochondrial ATP synthase, a participation of subunit I in dimerization of the chloroplast ATP synthase is likely. We expect the interaction to occur at the peripheral hydrophilic part of the subunit I (Fig. 6B), which is associated with the CF_1 complex.

Since the discovery of ATP synthase dimerization in mitochondria 6 years ago, the physiological relevance remains enigmatic. Dimerization of the mitochondrial ATP synthase has been connected to tasks such as protein complex stabilization [3], control of enzymatic activity [6,7] and modulation of the inner mitochondrial membrane structure [8,9], but there is still no proof for any of these hypotheses. All these functions may be of relevance for the dimerization of the chloroplast ATP synthase as well. Dimerization causes an about four times slower rotational diffusion of the ATP synthase in the bilayer and may increase the stability of the protein complex. In addition, the dimerization might influence the curvature of the bilayer and therefore be responsible for the formation of stroma thylakoids. Our model of the dimer (Fig. 6) is based on the stochastic rotation mechanism [43]. The ATP synthase couples vectorial proton translocation from the thylakoid lumen to the chloroplast stroma via the rotational movement of the oligomer III_{14}. Subunits γ and ε and the oligomer III_{14} together form a rotor. The stator consists of the $\alpha_3\beta_3$ hexamer connected to the membrane intrinsic subunit IV by the subunits I, II and δ. Revolution of the rotor relative to the stator drives the formation of ATP by inducing conformational changes within the catalytic hexamer $\alpha_3\beta_3$. Neither rotor nor stator is rigidly anchored in the lipid bilayer in a manner excluding their rotational movements in the monomeric state (Fig. 6A). In addition to ATP formation, a small portion of the rotational energy is transferred to the stator. As the stator is not fixed, a slow unidirectional rotation is induced. As a consequence, the rotation of the rotor relative to the stator is decelerated, and the energy inducing this rotation of the stator is lost for ATP synthesis. In dimers of the ATP synthase (Fig. 6B), associated by the peripheral stalk formed by subunits I and II, the unidirectional rotation of rotor and stator is hindered.

After all, the homology on the level of macromolecular assembly between the ATP synthase complexes of different biological origins seems to be higher than previously supposed. Dimerization, independent of its physiological significance, may be a general feature of all H^+-ATP synthase complexes.

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