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Characterisation of subunit III and its oligomer from spinach chloroplast ATP synthase

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Abstract

Proton ATP synthases carry out energy conversion in mitochondria, chloroplasts, and bacteria. A key element of the membrane integral motor CF_O in chloroplasts is the oligomer of subunit III: it converts the energy of a transmembrane electrochemical proton gradient into rotational movement. To enlighten prominent features of the structure–function relationship of subunit III from spinach chloroplasts, new isolation methods were established to obtain highly pure monomeric and oligomeric subunit III in milligram quantities. By Fourier-transform infrared (FTIR) and CD spectroscopy, the predominantly α-helical secondary structure of subunit III was demonstrated. For monomeric subunit III, a conformational change was observed when diluting the SDS-solubilized protein. Under the same conditions the conformation of the oligomer III did not change. A mass of 8003 Da for the monomeric subunit III was determined by MALDI mass spectrometry (MALDI-MS), showing that no posttranslational modifications occurred. By ionisation during MALDI-MS, the noncovalent homooligomer III_{14} disaggregated into its III monomers.

Keywords: Membrane protein; MALDI mass spectrometry; FTIR; CD spectroscopy

1. Introduction

The energy-converting processes of oxidative- and photophosphorylation generate a transmembrane electrochemical proton gradient across mitochondrial and chloroplastidic membranes, respectively. This gradient powers the synthesis of adenosine triphosphate via a membrane protein complex consisting of two rotating devices, the ATP synthase. The ATP-driven or generating engine, depending on the energetisation, is located in the hydrophilic F_1 portion of the ATP synthase. Chloroplast F_1 consists of the subunits $\alpha_3\beta_3\gamma\delta\varepsilon$ [1]. A rotating shaft composed of subunits γ and ε drives ATP formation by conformational changes of the catalytic hexamer $\alpha_3\beta_3$. The membrane-integrated part, CF_O, consisting of the protein subunits I, II, III, and IV functions as a rotary motor or proton pump [2,3].This device couples vectorial proton translocation to the rotational movement of the oligomer III_{14}, which is responsible for the rotation of γ and ε. While rotation in F_1 is well established, e.g. for chloroplast [4], the detection of a rotational movement in the membrane integral F_O sector was more difficult (e.g. Ref. [5]) and up to now not successfully proven for chloroplasts. In synthesis direction, the rotation of the c-oligomer has been demonstrated for a Na^+-translocating ATP synthase of *Propionigenium modestum* [6].While an X-ray crystallographic structure of incomplete yeast ATP synthase [7] displays a complex consisting of $\alpha_3\beta_3\gamma$ and c_{10} (homologous to III_{14}), much less is known about the chloroplast F_O. The two parts of ATP synthases are connected by two stalks, i.e., one central rotating shaft (γ, ε and III_{14}) and a thin stalk at the

Abbreviations: AFM, atomic force microscopy; CF_O, membrane integral part of chloroplast ATP synthase; DDM, *n*-dodecyl-β-D-maltoside; FTIR, Fourier-transform infrared; MALDI-TOF MS, matrix-assisted laser desorption/ionisation time-of-flight mass spectrometry

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periphery, which holds together the F_1 and F_O portion. The peripheral stalk, the stator, consists of the subunits δ, I and II.

The rotating oligomer III_{14} converts the electrochemical proton gradient into mechanical motion. Therefore, elucidation of the structure–function relationship of subunit III is of outstanding importance for bioenergetics. Electron micrographs show the overall arrangement of subunits in the chloroplast ATP synthase [8]. Several high-resolution structures are published for F_1 from mitochondria or *E. coli* [9–11]. X-ray structures of the CF_1 portion ($\alpha_3\beta_3\gamma$) have been obtained [12,13]. In contrast, the chloroplast F_O subunits are only poorly characterised. High-resolution structures of CF_O subunits are not available.

The topography and stoichiometry of the cylindrical III oligomer have been determined by atomic force microscopy (AFM) [3]. Single particle cryo-electron microscopy provided evidences for two membrane-spanning α-helices per subunit III monomer of spinach CF_O [14]. This agrees with studies of the monomeric bacterial subunit c, which has been investigated in organic solvents [15] or detergent [16]. While these structures differ in detail, they agree in showing that the c monomer is α-helical, forming a hairpin with a short loop. NMR studies suggest that the conformation of the c subunit of *E. coli* in organic solvent changes significantly in response to the protonation state of Asp61. In the functional ATP synthase complex, the III/c subunit forms a cylindical oligomer. A c-oligomer of 10 subunits was observed in a 4-Å X-ray structure of yeast ATP synthase [7]. 2D crystals of the c-subunit oligomer from *Ilyobacter tartaricus* revealed a similar cylindrical structure of 11 subunits [17] whereas AFM of 2D crystals of CF_O indicates a ring composed of 14 subunits [18].

The goal of the present study was to elucidate if oligomerisation of subunit III monomers results in conformational alterations. This is relevant not only for the interpretation of previous NMR and X-ray investigations [7,15,16,19] but also for the assembly of the membrane integral F_O proton turbine in vitro and in vivo. Isolation methods for the monomer III and the oligomer III_{14} were established, also with the aim to prepare samples for 2D and 3D crystallization. With CD and Fourier-transform infrared (FTIR) spectroscopy, it was found that they have a predominantly α-helical secondary structure, similar in the monomeric and oligomeric states. Depending on the solvent, differences in the secondary structure are observable. According to MALDI mass spectrometry (MALDI-MS) data, the assembled subunit III is not posttranslationally modified.

2. Methods

2.1. Isolation of CF_OF_1

CF_OF_1 was isolated from spinach chloroplasts by a modified procedure [2,20] of Pick and Racker [21] employing rate-zonal centrifugation as the last purification step. ATP synthase was obtained from the sucrose step gradient in 30 mM Tris, 30 mM succinate/NaOH pH 6.5, 0.5 mM EDTA, 1 mg/ml asolectin and about 30% sucrose, either in the presence of 0.2% Triton X-100 or 12 mM CHAPS as detergent.

2.2. Isolation of the monomeric subunit III

A method based on studies by Sigrist-Nelson et al. [22] was used to extract several milligrams of the subunit III from spinach chloroplasts [23]. Chloroplasts were isolated from spinach lettuce according to Apley et al. [24]. The chloroplast suspension was sedimented at $3640 \times g$ for 30 min, then homogenised in 120-ml chloroform/methanol/diethylether 2:1:12 (v/v/v) at a chlorophyll concentration of 1 mM. By centrifugation ($3640 \times g$, 5 min, RT), an occasionally occurring uppermost phase consisting of residual water was removed. The pellet containing subunit III together with the supernatant was diluted with the same volume of chloroform/methanol/diethylether 2:1:12 (v/v/v) and shaken vigorously. Upon centrifugation ($3640 \times g$, 5 min, RT) the supernatant was discarded and the pellet washed with 80-ml chloroform/methanol/diethylether 2:1:12 (v/v/v) and centrifuged again. The washing step was repeated with 80-, 60-, and 50- ml chloroform/methanol/diethylether 2:1:12 (v/v/v). The residual pellet was stirred in 50-ml chloroform/methanol 2:1 (v/v) for 64 h at 4 °C to solubilize subunit III. After centrifugation in Corex-tubes ($3000 \times g$, 5 min, 4 °C) to remove insoluble material, 6-ml 1-butanol was added to the supernatant. At 0.1 bar, most chloroform/methanol, but not 1-butanol, was removed for 4 h at RT using a rotating evaporator.

For a further chromatographic purification of subunit III, DEAE-cellulose was prepared as described by Sigrist-Nelson and Azzi [25]. The column ($d = 3.5$ cm, $h = 5.5$ cm) was equilibrated with 130-ml 1-butanol. The monomer III in 1-butanol was applied to the column. The solvents for chromatography were 60-ml 1-butanol, 60-ml 1-butanol/formic acid 50:1 (v/v), 50-ml chloroform/methanol 2:1 (v/v), and 50-ml chloroform/methanol/water 5:5:1 (v/v/v). Fractions of 10 ml were collected and stored at −20 °C. The column was regenerated with 30-ml chloroform/methanol/water 5:5:1 (v/v/v) with 22 mM ammonium acetate, and thereafter with 55-ml chloroform/methanol/water 5:5:1 (v/v/v) containing 47 mM ammonium acetate.

2.3. Isolation of the supramolecular complex III_{14}

Six-milliliter CF_OF_1 (about 24 mg) from sucrose gradient with Triton X-100 was dialysed against 1-l buffer A (10 mM Tricine/NaOH pH 7.8, 5 mM DTT) for 12 h at 4 °C using a visking type 8 (Biomol) dialysis tubing with a cut-off of 12 kDa. The dialysed CF_OF_1 solution was concentrated with an ultrafiltration unit (Centricon 100 kDa, Millipore) to a volume of maximal 4 ml. The ATP

synthase was mixed with solid SDS (final concentration 1% w/v) and incubated for 1 h at RT. Concentrated sample (0.5 ml) containing about 6-mg ATP synthase was loaded on a 12.4-ml linear gradient from 7.5% to 30% glycerol containing 10 mM Na-phosphate pH 7.8, 1% (w/v) SDS, or alternatively on a gradient buffer containing 10 mM Na-phosphate pH 7.8, 8 mM dodecyl maltoside (DDM). Other subunits besides III_{14} were separated by rate-zonal centrifugation at 222,500 × *g*, swing-out rotor, for 23 h at 20 °C when using the buffer with SDS, or at 10 °C when using the buffer with DDM. Fractions of 0.5 ml were collected from the bottom of the tube.

2.4. Protein analysis

SDS-PAGE and Coomassie R250 or silver staining [26,27] as well as protein determination were performed according to Ref. [20]. When determining the N-terminal protein sequence of subunit III, SDS-PAGE with borate buffer according to Poduslo [28] was employed. For sequencing, proteins were transferred after borate SDS-PAGE to PVDF-membranes (Bio-Rad) with the Bio-Rad transfer device Trans-Blot SD using the "semi-dry" technique [29]. Subunit III immobilized on the PVDF membrane was deformylated [30]. Membranes were stained with 0.1% (w/v) Coomassie R-250 in 50% (v/v) methanol and bands cut out with a scalpel. N-terminal sequencing was carried out by automated Edman degradation.

2.5. CD spectroscopy

CD spectra were recorded with a Jasco J-720 CD spectrometer at 4 °C and a cuvette of 0.1-mm path length. The secondary structure elements of the monomer III were calculated with the algorithm of Chang et al. [31], the helix content of the III_{14}-complex was calculated with the algorithm of Chang et al. [31] and Compton and Johnson [32]. To record the spectra, subunit III monomer was either used directly in organic solvent (chloroform/methanol 2:1 or 1-butanol) or after evaporation of the solvent transferred in aqueous solution of SDS. The oligomer III_{14} was measured in the density gradient solution with SDS.

2.6. FTIR spectroscopy

Measurements were made with a Bruker Vector 22 at RT and a CaF_2-cuvette of 10-μm path length. After evaporation of other organic solvents, 10 μl of the monomer of subunit III dissolved in $CDCl_3/CD_3OD/D_2O$ 4:4:1 (v/v) was deposited on the CaF_2 cuvette. Four hundred infrared spectra were recorded at an optical resolution of 2 cm^{-1}. The vibrational contributions of the solvent and of residual water vapor were subtracted from the spectrum of the dissolved protein. A sum of Gaussians was used to fit the amide I band of the IR spectrum as provided by the OPUS software (Bruker). Integration of each Gaussians provided the relative content of the respective secondary structure.

2.7. Mass spectrometry

Protein and peptide samples were analysed on a Voyager-DE PRO (Applied Biosystems) matrix-assisted laser desorption/ionisation time-of-flight mass spectrometer (MALDI-TOF MS). For peptide mass fingerprinting, tryptic peptides were obtained from silver-stained gels after destaining [33] and in-gel digestion [27]. The peptide mixtures were analysed using a 5 mg/ml solution of α-cyano-4-hydroxycinnamic acid in 50% acetonitrile/0.1% trifluoroacetic acid [34].

For molecular mass determination of the III-subunit, III_{14} solubilized in DDM was diluted to 0.15 mg/ml. A saturated solution of 2-(4-hydroxyphenylazo)-benzoic acid (HABA) in 50% acetonitrile/0.1% trifluoroacetic acid was used as matrix for dried droplet target preparation. Mass spectra were obtained in the linear, delayed extraction mode.

3. Results

3.1. Isolation of the subunit III monomer

Spinach chloroplasts were homogenised in chloroform/methanol/diethylether 2:1:12 (v/v/v), leaving the subunit III insoluble and removing the chlorophylls. Subunit III monomer was solubilized in chloroform/methanol 2:1 (v/v). No other protein species besides subunit III were solubilized as revealed by SDS-PAGE and silver staining (Fig. 1A). However, analysis by thin-layer chromatography showed that the protein contained lipid impurities and a small amount of pigments (data not shown). To remove the lipid and pigment contaminations, subunit III was further purified by chromatography on DEAE-cellulose. Pigments could be eluted in 1-butanol, or in 1-butanol/formic acid. The subunit III eluted in chloroform/methanol 2:1 preceding the main lipid impurity sulfoquinovosyldiacylglyceride (according to TLC) in chloroform/methanol/water 5:5:1 (v/v/v). The yield from the chloroplasts was 0.1 mg subunit III/mg chlorophyll before chromatography. Of the applied subunit III, 60% was recovered after chromatography.

3.2. Isolation of the oligomer III_{14}

CF_OF_1 was dissociated with 1% SDS into its subunits. We observed that most of the III_{14} oligomer remained intact at this SDS concentration in a temperature range of 15-35 °C. Dissociated proteins were separated by velocity sedimentation, then analysed by SDS-PAGE and silver staining. The supramolecular complex III_{14} sedimented fastest and was found at high purity in fractions 8–11 (corresponding to a glycerol concentration of 15.5–17.5%) as documented in Fig. 1B, lanes 7 and 8 (sharp band at about 100 kDa). To

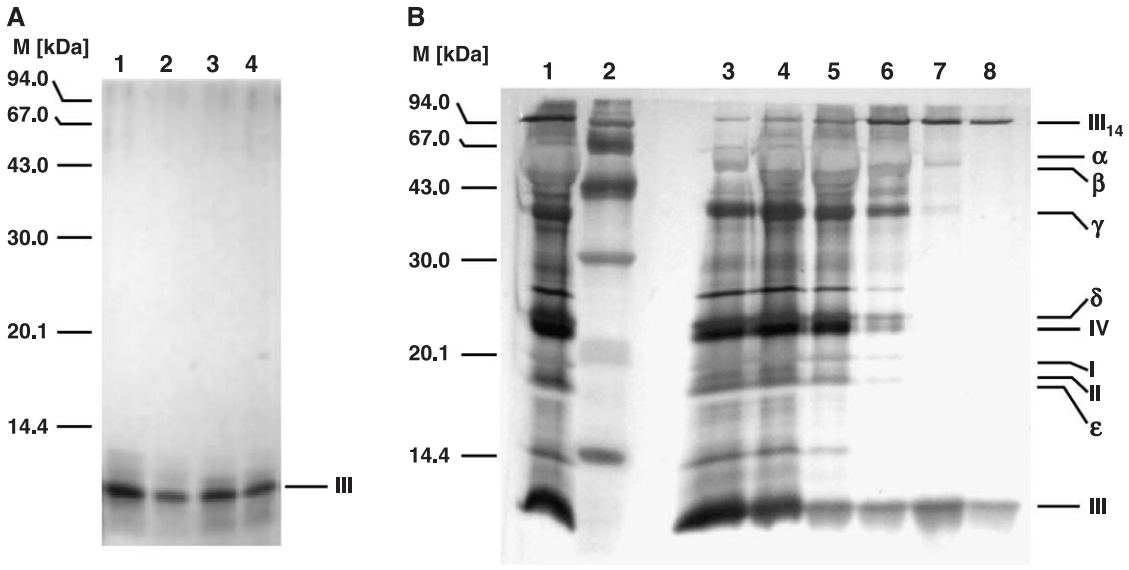

Fig. 1. Isolation of subunit III monomer and of the oligomer III_{14}. (A) By SDS-PAGE (T = 15%, C = 5%) with subsequent silver staining, subunit III extract before chromatography (lanes 1 and 2) and subunit III after chromatography (lanes 3 and 4) were analysed. (B) Isolation of the oligomer III_{14} by velocity sedimentation. Membrane proteins were analysed by SDS-PAGE (T = 14%, C = 4%) and silver staining. Lane 1: CF_OF_1 before sedimentation; lane 2: LMW-standard; lanes 3 to 8: fraction 1 (7.5% glycerol), fraction 3 (9.5% glycerol), fraction 5 (11.5% glycerol), fraction 7 (13.5% glycerol), fraction 9 (15.5% glycerol), fraction 11 (17.5% glycerol).

determine the degree of dissociation of the oligomeric subunit III, we analysed the bands of the oligomeric and the monomeric subunit on silver-stained SDS gels by densitometry and compared the areas using the software QuantiScan (Biosoft). Less than 20% of the oligomeric subunit III dissociated into the monomer. Besides the monomer and the oligomer III_{14}, no other stoichiometries were found. Partial dissociation of the complex may have occurred during isolation and storage or SDS-PAGE. If the gradient buffer contained SDS, 0.94-mg highly pure subunit III was obtained in fractions 8–11 from 25-mg CF_OF_1, which is a yield of about 22% of the subunit III originally present in the centrifuged ATP synthase. With DDM in the gradient buffer, the purest fractions with 14–18% glycerol contained 0.61-mg subunit III from 20-mg CF_OF_1 (a yield of about 15%). N-terminal sequencing of the first six amino acids (MNPLIA) after deformylation was used to confirm that the isolated protein corresponds to subunit III. The MALDI mass spectra of the isolated subunit III monomer and the oligomer show a single peak at a mass of 8003 Da, which is almost exactly the theoretical value of 8002.5 Da computed from the amino acid sequence (81 amino acids) and the formyl-modification of methionine (Fig. 2). This proves that no posttranslational modifications of subunit III occurred. No impurities were present. The identification of subunit III by tryptic digestion and MALDI mass spectrometry of the peptide fragments verified the result of the N-terminal sequencing. Fragments of 1027.55 and 3728.03 Da were detected. Theoretically, six fragments should result from tryptic digestion of subunit III, but the low and high (>4000 Da) mass of the other fragments makes them difficult to identify. The mass of the identified fragments corresponds to the theoretical values of 1027.55 and 3728.00. The latter represents the N-terminal peptide including the formyl modification.

3.3. Secondary structure of subunit III

The subunit III monomer was obtained by solubilisation and chromatography in chloroform/methanol 2:1. To record the CD spectrum, the organic solvent was evaporated and the protein was resuspended in aqueous 2% (w/v) SDS. The CD spectrum (Fig. 3A) of the subunit III monomer displays the double minimum of the transitions at 208 and 222 nm, characteristics of α-helices. This was confirmed by calculating the contribution of each protein secondary structure element to the spectrum by the algorithm of Chang et al. [31]: 44% α-helix, 26% β-sheet, 25% random coil.

To study the influence of the solvent on the conformation of the protein, a spectrum in 1-butanol was recorded from 191 to 260 nm. With chloroform/methanol 2:1 as solvent, the CD spectrum could only be recorded in the range between 215 and 260 nm. In the measured wavelength ranges, the CD spectra of subunit III monomer in chloroform/methanol 2:1 and 1-butanol (data not shown) were identical to the spectrum in 2% SDS, indicating that the overall structure of the III is neither affected by the two different solvents nor altered in the more lipid-like SDS micelle.

The IR spectrum of the monomer III dissolved in $CDCl_3$/CD_3OD/D_2O 4:4:1 (v/v) (Fig. 3B) displays four prominent bands. The amide I band (mainly C = O stretching mode of the protein backbone) appears at 1656 cm^{-1} with a shoulder at 1628 cm^{-1}. The former frequency confirms the predominance of the α-helical conformation of the protein

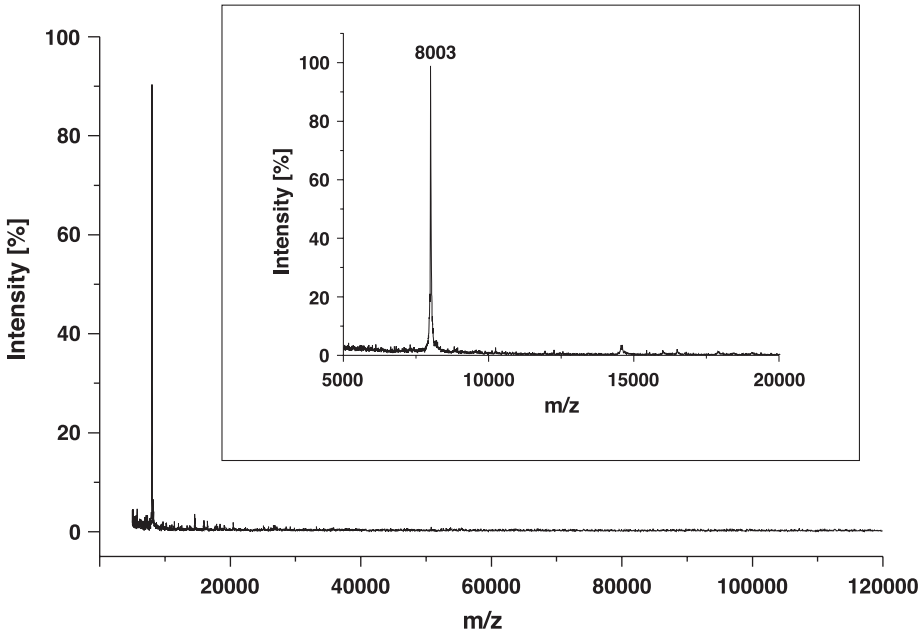

Fig. 2. MALDI-TOF mass spectrum of subunit III oligomer, displaying the *m*/*z* of the dissociated monomeric subunit III.

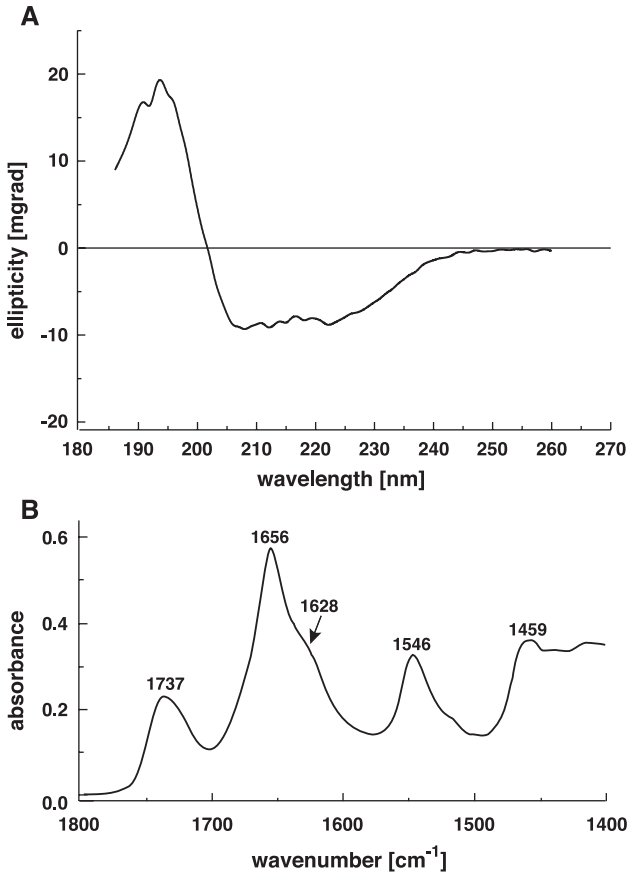

Fig. 3. CD spectrum (A: 184–260 nm) and FTIR spectrum (B: 1800 to 1400 cm^{-1}) of isolated subunit III monomer. To record the CD spectrum, the subunit III monomer after evaporation of other organic solvents was resuspended in aqueous 2% (w/v) SDS. For recording the FTIR spectrum, the monomer III was dissolved in $CDCl_3/CD_3OD/D_2O$ 4:4:1 (v/v).

backbone. The peak at 1546 cm^{-1} corresponds to the amide II vibration (C=N stretch coupled to the N–H bending mode of the backbone). This band indicates that the backbone protons do not exchange with deuterons in the deuterated solvent. The corresponding amide II′ mode appears at 1459 cm^{-1}, typically downshifted by ca. 100 cm^{-1}. To calculate the proportion of each secondary structure element from the FTIR spectrum, the amide I absorption band was fitted to a sum of Gaussians. According to this analysis, subunit III is predominantly α-helical (50% α-helix, 30% β-sheet, 20% random coil) consistent with results from CD spectroscopy. Not only the α-helix but also the proportion of the two other secondary structural elements coincide quite well for both methods. Nevertheless, the high proportion of β-sheet is in contradiction to the recent electron and X-ray structures [7,16].

The band at 1737 cm^{-1} in the FTIR spectrum of the monomeric subunit III is caused by residual lipid from the buffer which was not completely removed during the purification procedure.

3.4. Comparison of subunit III monomer and oligomer

For a direct structural comparison of the subunit III oligomer with the monomeric subunit, a sample of the oligomer was incubated with chloroform. This treatment dissociates the oligomer into monomers (data not shown). After evaporation of the organic solvent, subunit III monomer was dissolved in 0.5% or 1% SDS buffer. The CD spectra of the samples prior or after chloroform treatment show the same secondary structure of the isolated monomer and the oligomer in the presence of 1% SDS (Fig. 4). The CD spectra of the subunit III oligomer in 0.5% and 1% SDS

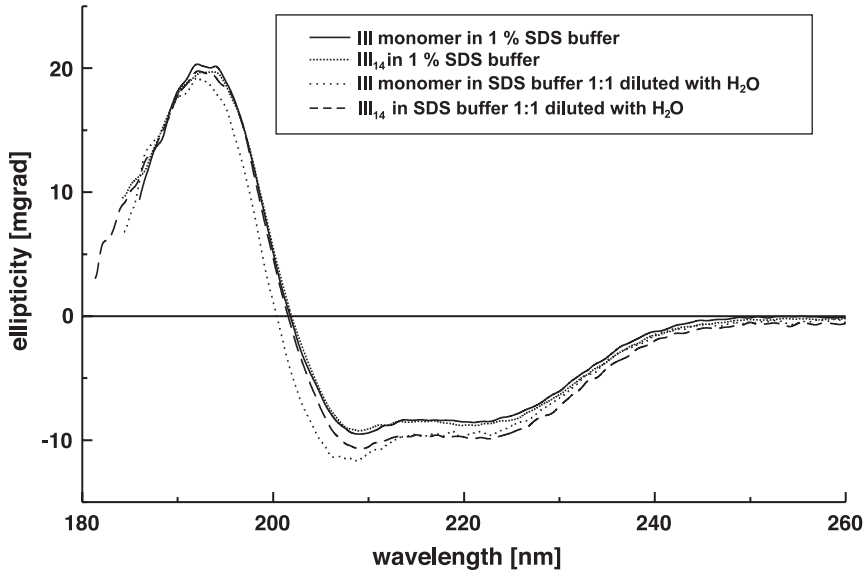

Fig. 4. CD spectra of oligomeric and monomeric subunit III, recorded in 1% and 0.5% SDS buffer, respectively. The spectra were normalized to the protein mass. To obtain the monomer III, the oligomer was dissociated by chloroform treatment.

buffer suggest a higher α-helix content at the 50% reduced SDS concentration. However, significantly larger differences exist between the CD spectra of the III monomer in 1% SDS and 0.5% SDS, as exemplified by the pronounced hypsochromic shift at the lower concentration. If the proportion of α-helix for the oligomer in 1% SDS is set to 1.0, the relative content for the monomer is 0.9 in 1% SDS and only about 0.7 in 0.5% SDS. In summary, in 1% SDS, the helix contents of the subunit III monomer and oligomer are very similar. However, 1:1 dilution of the detergent leads to a drastic decrease of the α-helix content for the III monomer only, indicating that the conformation of the subunit III monomer is stabilized by protein–protein interactions in the oligomer.

4. Discussion

The lack of structural information for chloroplast F_O motivated us to develop new isolation procedures for monomeric subunit III and its oligomer III_{14}. Previous methods for isolation of the oligomer from spinach chloroplasts had two main disadvantages. Both methods [2,35] were based on gel electrophoresis. Therefore, it was necessary to extract the protein from the gel matrix for further use. Only small amounts of protein could be obtained by both methods, insufficient for screening of 2D and 3D crystallization conditions. The newly established method with SDS in the buffer for the density gradient allowed us to isolate a highly pure oligomer III in milligram amounts. Upon replacing the detergent SDS by DDM, the oligomer was contaminated with the α, β, and γ subunits of CF_1. This latter detergent was therefore inefficient in dissociating the ATP synthase. However, it was preserving the oligomeric architure better, so that the amount of contaminating monomer was lower and the oligomer was more stable. It is worth noting that isolation of the oligomer c_{11} of *I. tartaricus* [17] and of yeast F_1c_{10} [7] has been described but not for *E. coli*, even though an in vitro self-assembly of monomeric subunits c into an oligomeric ring is possible in *E. coli* [36]. Compared to mitochondria or *E. coli*, the oligomers from spinach chloroplasts and *I. tartaricus* [37] are very stable, because both can be found in the high mass range of SDS gels.

In order to determine the secondary structure of subunit III, CD and FTIR spectroscopy were applied. Both samples, i.e., subunit III and its oligomer, have a predominantly α-helical structure. The determined helix content of about 50% seems to be too low for a protein arranged as an α-helical hairpin. But indeed, the value is in accordance with the helix content of about 52% in the NMR structure of subunit c from *E. coli*, which possess three helical segments [15]. In *P. modestum*, the α-helix content of subunit c is much higher (about 80%) in the NMR structure [16], but the earlier published value of 40% based on CD measurement [38] is in line with our own results. For subunit c of *Neurospora crassa*, a helix content of 60% in SDS has been determined by CD [39]. The helix content of subunit III or c is not constant, but changes with the solvent or pH. In *E. coli* a significant change in the conformation of the c subunit was observed after pH increase from 5 to 8 in organic solvent/monomer c mixtures [19]. This conformational alteration was suggested to be an essential mechanism in subunit c to transfer protons to subunit a. For mitochondrial subunit c, a conformational change with different solvents has been reported [39]. Differences of about 20% in the helix content occurred upon changing the detergent from SDS to octyl glucoside. In organic solvent or in the presence of non-

charged detergent, the helix content is markably higher than in 0.1% SDS. In the present study, for monomeric subunit III, a conformational change is observed upon dilution of the SDS solubilized protein. We conclude that a pH change [19] or a change in the hydrophobicity of the environment can induce conformational changes in the monomeric subunit III or c, but smaller changes occur in the oligomer. As a result of this conformational alteration, protons might be transferred from Asp 61 or Glu 61 of subunit c (III) to subunit a (IV). In the oligomeric subunit, solvent-induced conformational changes are less pronounced (Fig. 4). Since during the rotation of the proton turbine only one of the protomers is protonated at any time according to the presently postulated mechanism [1,19], the possible changed helix content of only one subunit cannot be resolved when observing the whole oligomeric cylinder with CD or FTIR spectroscopy. Besides the α-helix content in CD or FTIR spectra, a significant proportion of β-sheets is detected. This is in contradiction to the more direct biophysical methods (NMR, electron microscopy, and X-ray diffraction), in which no β-sheets were revealed. In NMR studies often an isotropic organic solvent was used [15], which might perturb the secondary structure much more than the aqueous buffers in our study. However, the main cause for the discrepancy is most probably that CD and FTIR only reflect the overall structure but do not allow precise determinations of the exact secondary structure.

Application of MALDI mass spectrometry for integral membrane proteins is still a challenge although the first successful attempts emerged in 1995 [40]. With MALDI-MS we could determine the mass of the extraordinary hydrophobic (GRAVY index 1.035) monomeric subunit III and were able to reveal that besides the formyl-modification, no posttranslational modifications occurred. Additionally, analysing the band of the subunit III oligomer from SDS-PAGE by MALDI MS, we were able to prove that this band contains subunit III only. No other proteins were present as contaminants. The subunit III monomer of spinach (81 amino acids) has a lower molecular mass (8003 Da) than its homologue from *Chlamydomonas reinhardtii* (8125 Da, 82 amino acids, Meyer zu Tittingdorf, submitted for publication), and from *I. tartaricus* (8790 Da, 89 amino acids, [17]). Two main problems emerge with mass spectrometric identification of this subunit. The first problem is the small size. Only two tryptic fragments could be obtained, therefore the data base search has to be very restricted. The second problem is the lack or limited accessibility of proteolytic cleavage sites in the membrane-spanning segments [41].

According to Fig. 2, only a single ion of $m/z = 8003$ is present in the MALDI spectrum of the III_{14} sample. This shows, on the one hand, the very high purity of the preparation. On the other hand, since the starting material was the III_{14} oligomer, the present time limitation of MALDI-MS in the investigation of protein complexes stabilized by only hydrophobic interactions is documented. The use of organic solvents during sample preparation affects the hydrophobic protein–protein interactions. Also the matrix employed or the high-energy laser ionisation might dissociate the oligomer into its protomers. As long as appropriate matrices or ionisation procedures, as successfully applied in the case of the trimeric porin channel [40], are not available for other noncovalent protein complexes, such as CF_OF_1, biochemical cross-linking of the various subunits might be the only way to determine their mass by MALDI-MS.

The highly pure samples of monomeric and oligomeric subunit III now available, as well as their determined secondary structures and solvent-induced conformational changes, will hopefully facilitate the elucidation of the structure as well as of the mechanism of the chloroplast proton ATP synthase.

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