Bacterial F-type ATP synthases follow a well-choreographed assembly pathway

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Abstract

F-type ATP synthases are multiprotein complexes composed of two separate coupled motors (F_1 and F_0) generating adenosine triphosphate (ATP) as the universal major energy source in a variety of relevant biological processes in mitochondria, bacteria and chloroplasts. In the past decades, ATP synthases have become a subject of high interest, as a target for therapeutic use in the treatment of a variety of diseases. While the structure of many ATPases is solved today, the precise assembly pathway of F_1F_0 -ATP synthases is mostly still unclear. To probe the bacterial F_1 assembly of *Acetobacterium woodii*, we studied the self-assembly of purified proteins under different environments. We report assembly requirements, important assembly intermediates *in vitro* and *in vivo*, the crucial role of nucleotide binding (as opposed to ATP hydrolysis) and correlate results with complex activity. Finally, we propose a model for the assembly pathway for the formation of a functional F_1 complex.

Introduction

Life needs energy for numerous biochemical energy-consuming processes to maintain cellular functions, such as biosynthesis, membrane transport, regulatory networks and nerve conduction. The energy is provided in the form of ATP which cells have to convert using external energy sources such as light or nutrients. ATP synthases, the molecular machines employed for such ATP production are omnipresent and found in many biological systems, such as the inner membrane of mitochondria, the thylakoid membrane of chloroplasts and the bacterial plasma membrane and therefore of high importance. Recently, the F-type ATP synthase has been highlighted as a novel drug target against *Mycobacterium tuberculosis*^[1,2] and the enzyme is gaining more and more interest as an attractive molecular target for the therapy of a variety of diseases (e.g. neuropathy, ataxia, retinitis pigmentosa syndrome, the familial bilateral striatal necrosis, one form of Leigh syndrome and Alzheimer's disease)^[3–5]. These macromolecular complexes consist of a hydrophilic chemical-driven F_1 - and the ion-driven membrane embedded F_0 -module, as shown in Fig 1d. ATP is synthesized from adenosine diphosphate (ADP) and inorganic phosphate (P_i) in the six nucleotide binding sites situated in the interface of the α and β subunits in the soluble hexameric F_1 head. The energy is provided via a rotary mechanism of the membrane-bound F_0 part driven by the electrochemical gradient across the membrane via channels at the interface of the peripheral stator and the c-ring causing the c-ring to rotate^[6–8]. Working in reverse direction the ATPase/synthase can also hydrolyze ATP and function as an ion pump, dependent on the physiological demand of the living cell^[7,9,10].

The ATPase F_1 part consists of nine subunits arranged as δ atop the hexameric head $\alpha_3\beta_3$, which sits on a central stalk $\gamma\epsilon$, while the membrane integrated F_0 part comprises the peripheral stator unit ab_2 and a membrane-embedded c-ring (Figure 1). The c-ring is known to vary between different organisms, so far c-subunit stoichiometries between 9 and 15 have been reported^[11–15]. The central stalk $\gamma\epsilon$ of F_1 interacts with the c-ring as well as the hexameric head. The torque of γ leads to conformational changes in the β -

subunits causing different nucleotide binding affinities (termed open, loose, closed and tight conformation) resulting in either ATP synthesis or hydrolysis^[22,23]. For *E. coli* the subunit ϵ was found to be critically important for the integration of the soluble part F_1 with the membrane part F_0 ^[16–18]. It is as well known to exert a regulatory effect on bacterial ATPase activity^[19–21].

As a diversity of diseases are related to the function of F_1F_0 -ATP synthases^[3–5], research aimed not only at the structure but the assembly process of ATP synthases could play an essential role in the development of treatments. Here we investigate the assembly process of bacterial F-type ATPases, for the example of *Acetobacterium woodii* (*A. woodii*). *A. woodii* has a sodium driven Na^+ - F_1F_0 -ATPase and an unusual c-ring comprised of two different types of subunits, for which mass spectrometric analysis with Laser Induced Liquid Bead Ion Desorption (LILBID) has revealed a stoichiometry of 9:1 ($c_{2/3}$: c_1)^[24].

The assembly of this hybrid rotor has been shown to require the assembly factor AtpI, which could not be replaced by analogue *E. coli* AtpI^[25]. Additionally studies have shown the assembly of bacterial F_0 to depend on the signal reconstitution particle pathway (SRP), SecYEG translocon and YidC, which are required for the co-translational insertion of subunits a and b^[26]. While c-subunits and subunit b can be co-translationally reconstituted in the membrane independently of subunit a, the stable insertion of subunit a requires the presence of subunits b and c^[27].

The bacterial F_1 module $\alpha_3\beta_3\gamma\epsilon$ assembles independently of the membrane-bound F_0 part^[7,28]. *In vitro* reconstitution of isolated α -, β - and γ -subunits support the possibility of *in vitro* formation of a functional active $\alpha_3\beta_3\gamma$ -subcomplex^[29,30]. However, detailed studies of the assembly of bacterial F_1 from single subunits are still missing. As two F_1 -specific chaperones Atp11p and Atp12p are known to be functionally relevant for the assembly and solubility of β and α ^[31] in mitochondria, while no such chaperones are identified in bacteria, the question arises if chaperones are relevant for the bacterial F_1 assembly.

Methods

Cloning expression vectors of the *A. woodii* F_1 ATP synthase

Using pKB3-His (a pET21a[+] vector derivative) as a master plasmid, which was kindly provided by Karsten Brandt (Müller laboratory^[25] at Goethe University), we performed PCR for cloning of deletions or point mutations and ligation independent cloning with the In-Fusion HD Cloning Kit (Takara Bio). For cloning of single genes encoding for subunits and subcomplexes of F_1 (*atpHAGDC* for subunit δ , α , γ , β and ϵ) with an N- or C-terminal tag (StrepI- or His₆-tag) were cloned into a commercially available pET21a(+)-vector. All generated plasmids are listed in Supplemental Information (Table S1), respectively. A list of primers, used for cloning the respective plasmids are given in Supplemental Information (Table 2). Stellar competent cells (Takara Bio) were used for plasmid amplification. All constructs including mutations were verified by sequencing (Microsynth SeqLab).

Purification of heterologous F₁ ATP synthase subunits and subcomplexes of *A. woodii* containing a His₆-tag or a Strep-tag

All constructs were transformed in *E. coli* BL21gold(DE3) competent cells (AgilentTechnologies) following the provided manual. LB Agar (Lennox) transformation plates supplemented with 100 µg/mL ampicillin were used for the transformation of all constructs. Cell colonies were grown at 37 °C overnight and stored at 4°C up to two weeks. A single clone was picked to inoculate a pre-culture of Lysogeny Broth (LB) containing the respective antibiotic and was grown at 37 °C and 150 rpm overnight. Pre-culture was inoculated in main culture of Terrific Broth (TB) medium supplemented with respective antibiotic. Cells were cultivated at 37 °C and 150 rpm until an OD_{600nm} of 0.5-0.7 was reached. After induction the main culture with 0.25 mM Isopropyl-β-D-thiogalactopyranosid (IPTG) the protein expression was performed at 20 °C and 150 rpm overnight. The cells were harvested by centrifugation at 4.000 rcf for 20 min at 4 °C. The isolated cell pellets were resuspended in 20 mL wash buffer I (50 mM KP_i, 200 mM NaCl, 20 mM imidazole, 10 % glycerol, pH 7.4) supplemented with 1 mM EDTA and DNase I. Using a French Pressure Cell Press cells were mechanical disrupted at a pressure of 700 bar and cell debris was removed by centrifugation (50.000 rcf for 60 min at 4 °C). After the addition of 2 mM MgCl₂ cell lysate was incubated with 5 mL Ni-NTA Agarose (binding capacity 50 mg/mL) (MACHEREY-NAGEL) for 30 min. Column was washed with 5 column volumes wash buffer to remove unbound proteins and bound protein was eluted with 2.5 column volumes wash buffer containing 300 mM imidazole. The elution fractions were analyzed with SDS-PAGE, concentrated with an Amicon Ultra centrifugal filters and purified by size exclusion chromatography (SEC) with a Superdex 75 10/300 (GE-Healthcare). Protein samples were shock frozen in liquid nitrogen and stored at -80 °C. Same workflow procedure were performed for all protein constructs which contained a StrepI-tag with the exception of using a 5 mL Strep-Tactin-column (binding capacity 15 mg/mL resin) (Iba) with the respective wash buffer II (50 mM KP_i, 200 mM NaCl, 10 % glycerol, pH 7.4) and elution with the wash buffer II containing 2.5 mM d-Desthiobiotin.

In vitro assembly and mass spectrometric analysis

For the *in vitro* studies all in *E. coli* heterologously purified subunits α, β, δ and γε were incubated in a ratio of 3:3:1:1 for assembly into the full F₁-complex, or subsets for subcomplexes in 50 mM KP_i, 200mM NaCl, 2 mM ATP, 2 mM MgCl₂, 10 % (v/v) glycerol, pH 7.4 at 4°C for 1 hour. For LILBID-MS measurements the buffer was exchanged to 100 mM ammonium acetate, 2 mM ATP, 2 mM MgCl₂, pH 7.4 at 4 °C before measurements using desalting columns with a cut-off 7 kDa (Zeba Micro Spin Desalting Columns, Thermo Scientific). Approximately 5 µL sample were used per measurement. LILBID-MS droplets were generated by a piezo-driven droplet generator (MD-K-130, Microdrop Technologies GmbH, Germany). This generator produces droplets of 50 µm diameter with a frequency of 10 Hz. The droplets were transferred to vacuum and irradiated by an IR laser at 2.94 µm, a vibrational absorption wavelength of water, which leads to the explosive expansion of the sample droplet. The released ions then are analysed using a home-built time-of-flight spectrometer^[32]. The LILBID ion source was run at standard settings. The

presented spectra show the averaged signals of several hundred up to thousand droplets. Data processing was done using the software Massign^[33].

Electrospray ionization mass spectrometry (ESI-MS) was performed on a Synapt G2S (Waters Corp., Wilmslow, Manchester, UK) equipped with a high mass quadrupole upgrade. Pd/Pt sputtered nESI tips were pulled in house from borosilateglass capillaries on a Flaming/Brown Micropipette Puller (P-1000; Sutter Instrument Co.). For the analysis of purified F_1 -subunits, capillary and cone voltages were set to 1.85 kV and 150 V, respectively. The source block temperature was set to 30°C. Neither trap nor transfer collision cell was used. Directly prior to MS analysis, 10 μ L of the protein solution (30 μ M) was buffer exchanged into 100 mM ammonium acetate, 100 μ M ATP, 100 μ M $MgCl_2$ and pH 7.4 at 4 °C using Zeba Micro Spin Desalting Columns (Thermo Scientific).

For the ion mobility MS experiments, $\alpha\beta$ heterodimers were analyzed in positive ion mode using a capillary voltage of 2.1 kV. The rest of the settings for MS analysis were adjusted as following: cone voltage 100 V at an offset of 150 V, 20 °C source temperature. The instrument was calibrated by a conventional Csl solution. Ion mobility (IM) experiments were done using a traveling wave setup operating at a wave height of 40 V, a travelling wave velocity of 700 m/s, a nitrogen gas flow of 90 mL/min and a drift cell pressure of 3.5 mbar. MS-MS was performed to isolate the $\alpha\beta$ heterodimers, which were selected at $m/z=4950$ using an LM resolution of 12 and a HM resolution of 15. Collision induced unfolding (CIU) experiments were performed by increasing the trap collision energy (CE) in steps of 5 V from 85 to 200 V. Data analysis of ESI-MS and IMS experiments was done using the software MassLynx V4.1, TWIMExtract^[34], CIUSuite^[35] and UniDec^[36].

ATP hydrolysis assay

The enzyme-coupled activity assay performed in this thesis was adapted from references^[37,38]. Generally, assays were run in 96-well f-bottom microtiter plates (Greiner Bio-One). The ATP hydrolysis assay was performed to analyze the ATP hydrolysis activity of the single F_1 -subunits, the *in vitro* assembled complexes of F_1 and *in vivo* purified F_1 -subcomplexes. During this assay ATP was constantly regenerated by an enzyme-coupled reaction, while the oxidation of NADH was spectroscopically followed via the decrease of absorbance at 340 nm. Measurements were performed in for subunits and *in vitro* assembled subcomplexes, for which the following amounts of the respective proteins were used: 5.5 μ M in case of α and β , 1.8 μ M $\gamma\epsilon$, 5 μ M ϵ . In the enzymatic assays of *in vitro* complexes 5.5 μ M protein was used for α as well as β and 1.8 μ M $\gamma\epsilon$ or 5 μ M of the ϵ -subunit respectively, to allow for subunit stoichiometries of 1:1 for the $\alpha\beta$ heterodimer, and 3:3:1 for $\alpha_3\beta_3\gamma\epsilon$. *In vivo* produced $\alpha\beta$ and $\alpha_3\beta_3\gamma\epsilon^*$ were employed with 0.5 μ M and 0.25 μ M, respectively.

Protein solutions were diluted to 100 μ L total volume (100 mM TRIS, 100 mM maleic acid, pH 7.5, 5 mM $MgCl_2$, 3 mM phosphoenolpyruvate (PEP), 4 mM ATP, 0.5 mM NADH, 10 units L-lactate dehydrogenase (L-LDH), 10 units pyruvate kinase (PK)), filtered and degassed for 700 s in 3 s intervals at 25 °C. The decrease in absorbance at 340 nm per second was determined by fitting a linear fit of the slope. All

measurements were performed in biological triplicates except $\alpha_3\beta_3\gamma\epsilon^*$ with two technical duplicates. Kinetic data was analyzed with Origin 2018.

High performance liquid chromatography (HPLC) analysis

For the assembly studies and chromatographic separations 60 μg protein was applied on a SEC column (bioZen 1,8 μm SEC-3) coupled with UV detection in the elution buffer A (50 mM KPi , 200 mM NaCl, pH 7.4) or elution buffer B (50 mM KPi , 200 mM NaCl, 2 mM ATP, 2 mM MgCl_2 , pH 7.4) at 4 °C, respectively.

Results

Role of ATP for the *in vitro* assembly process of bacterial F-type ATP synthases. Since the assembly mechanism of the bacterial F-type ATP synthase, in particular for the soluble F_1 module, is mostly still unclear, we aim to shed light on this process. We determine individual subunit interactions, relevant conditions and also investigate whether the assembly of all different subunits follows a specific order.

In a first step, we purified recombinant subunits (α , β , δ and $\gamma\epsilon$) of the soluble part F_1 individually. Attempts to express the γ -subunit in *E. coli* separately yielded insoluble aggregates (inclusion bodies). Therefore, we cloned the genes *atpG* and *atpC* bicistronically in the expression vector and purified the central stalk $\gamma\epsilon$ to generate a higher complex stability and protein solubility (Fig. S7). To investigate individual assembly steps we incubated different subunit combinations under different assembly conditions and determined with LILBID-MS and HPLC experiments which complexes did or did not form. The resulting (sub)complexes represent key steps in the assembly process. To ensure the relevance of the observed *in vitro* subcomplexes for the *in vivo* processes in the cell and for comparison of structure, complex stability and bioactivity, we then set out to produce homologous subcomplexes *in vivo*.

Incubation of recombinant α and β *in vitro* in ammonium acetate buffer without any additives shows no specific α/β -oligomerization into higher subcomplexes (Fig. 1a) suggesting that our experiment is missing something essential. The cytoplasmic concentrations of ATP in active cells is known to be approximately in the range of 2-5 mM^[39] which prompted us to test the relevance of ATP for this assembly. As MS resolution can be affected by additives, we stayed in the lower range of the native concentration and incubated the same proteins with 2 mM ATP and MgCl_2 (Fig 1). (Effect of ATP/ Mg^{2+} on LILBID-MS spectra is shown in Supplemental Information Figure S1). Fig. 1b demonstrates that the addition of ATP/ Mg^{2+} leads to specific $\alpha\beta$ heterodimer formation only (no unspecific homodimers) with a charge state distribution (-1 to -3). Interestingly, no higher α/β subspecies can be identified, even though ATPases are known to form a hexameric head, containing three α - and β -subunits, respectively. As *in vivo* comparison, we purified and isolated an $\alpha\beta$ complex from *E. coli*, which we then analyzed under the same *in vitro* conditions. The *in vivo* $\alpha\beta$ complex showed the same heterodimer and no higher oligomeric states either (Fig. S2). It should be noted that the *in vitro* formed $\alpha\beta$ heterodimers need the presence of ATP/ Mg^{2+} not only for formation but in order to remain stable, as can be seen from measurements, for which the buffer is slowly diluted during the LILBID-MS run (Fig. S1). Reduction of the ATP/ Mg^{2+}

concentration during LILBID-MS goes along with an increase in the signal resolution but reduced complex stability. Interestingly the *in vivo* $\alpha\beta$ complex seems to suffer less from reduced ATP/Mg²⁺ concentration (Fig. S2), which is the first hint that assembly of α and β *in vitro* and *in vivo* might lead to slightly different heterodimers.

For comparison with the LILBID-MS results we investigate the *in vitro* assembly of subunit α and β by an alternative analytical method: size exclusion chromatography (SEC) by HPLC, using the SEC-column with a protein separation range of 10k-700kDa (Fig. 1C). The chromatograms show a shift into a shorter elution peak time confirming $\alpha\beta$ heterodimer formation only upon addition of mM ATP and MgCl₂. LILBID and SEC experiments both show (Fig. 1a-c) that the additives ATP/Mg²⁺ are crucial for the *in vitro* assembly of subunits α and β of Na⁺-F₁F₀- ATP synthases of *A. woodii*.

Assembly studies of single isolated subunits with next neighbors

α , β , δ and $\gamma\epsilon$ Figure 2 gives an overview of all possible subcomplexes which we observed after incubation of different combinations of subunits. This provides information about next neighbor interactions and order of assembly steps within the F₁- complex. As mentioned above we can observe $\alpha\beta$ heterodimer assembly under the right assembly conditions – but no higher order oligomers, if just these two subunits are present (Fig. 1b). Similarly, we observe that upon incubating either subunit α or β with the $\gamma\epsilon$ complex, a single subunit α or β can bind to the $\gamma\epsilon$ complex, forming $\alpha\gamma\epsilon$ or $\beta\gamma\epsilon$ complexes, respectively. These complexes are seen under soft laser conditions. Already medium laser desorption conditions, allow the ϵ -subunit to dissociate from the $\gamma\epsilon$ complex (Fig. S8c-d), giving rise to $\alpha\gamma$ or $\beta\gamma$ complexes. This shows that α or β bind to the g-subunit, while the ϵ -subunit is only weakly bound to g and not directly involved in the interaction with α or β .

As subunit α and β alone can only form heterodimers and only a single α or β can bind to γ if incubated alone, the next step was to purify and incubate recombinant α , β and $\gamma\epsilon$ in presence of 2 mM ATP / MgCl₂. Mass spectrometric analysis showed the formation of the $\alpha_3\beta_3\gamma\epsilon$ -subcomplex which we could identify at a mass of 378.72 kDa (theoretical protein mass 378.24 kDa) and a charge state distribution from -1 to -5 (Fig. 3). In contrast, no higher subcomplex formation was observed if the same experiment was performed without ATP/Mg²⁺. This implies that these additives are vital for the entire *in vitro* reconstitution process providing complex stability.

Including the δ subunit to the incubation then led to its association with the preformed $\alpha_3\beta_3\gamma\epsilon$ complex resulting in the fully assembled F₁-complex $\alpha_3\beta_3\delta\gamma\epsilon$ with a determined protein mass of 399.84 kDa (theoretical mass: 399.78 kDa) and a charge state distribution of -1 to -3 (Fig. 3d). The spectrum shows the fully assembled F₁-complex, as well as subcomplexes $\alpha_2\beta_2\gamma\epsilon$, $\alpha\beta\gamma\epsilon$ and $\alpha\beta\gamma$, which could stem from incomplete assembly or dissociation. Surprisingly our spectra show no subcomplexes with an odd number of α or β subunits, indicating that the $\alpha\beta$ heterodimer is the stable basic binding block from which the hexamer is build up. No δ is seen binding to any of the single subunits, which is in line with studies

showing interaction of δ with α -subunits only if complexed with other F_1 -subunits^[40]. No δ binding is seen for any of the mentioned subcomplexes either (Fig. 2a and Fig. S8a-b), suggesting the fully assembled $\alpha\beta$ -hexamer to be a prerequisite for binding of δ . Combined the observed on-pathway subcomplexes en-route to the F_1 complexes allow to propose the ATPase assembly to occur only via the binding of preformed $\alpha\beta$ heterodimers onto the γ -subunit, to form the hexameric head, which then allows for binding of δ . As γ could not be purified separately we could not determine in the same manner, if ϵ is a prerequisite for binding of the $\alpha\beta$ dimers onto γ .

For comparison we then attempted to purify and isolate all proposed on-pathway subcomplexes *in vivo*. All expected complexes (Fig. 2c) could be purified. As complexes lacking ϵ could be achieved, we conclude that the hexameric head forms around γ independently of ϵ . Mass spectrometric analysis (Fig. S6) reveals, as for the *in vitro* complexes, only complexes with the same amount of α and β subunits, supporting the $\alpha\beta$ heterodimer as a basic building block. Incubation of δ with the *in vivo* complex as well confirms our *in vitro* results, which showed δ to only bind onto the complete $\alpha\beta$ hexamer.

Interestingly, the *in vivo* $\alpha_3\beta_3\gamma\epsilon$ complex (Fig. S6) shows a significantly higher complex stability even without addition of ATP/Mg²⁺ than the *in vitro* complex (Fig S2e-f). This difference could be very interesting, as it might show that there are deviations in the *in vivo* and *in vitro* assembly, possibly due to structural differences of the proteins. An alternative explanation would be steric hindrances due to the tags, which are part of our *in vitro* purified proteins. The *in vitro* studies were performed on N-terminal Strept- α and His₆- β , C-terminal His₆- δ and $\gamma\epsilon$ (His₆-tag located on subunit ϵ), while the *in vivo* complex was expressed with only one N-terminal His₆-tag on the β -subunit. For comparison we purified a complex, which includes the same tags on every subunit as our isolated purified subunits, which we name $\alpha_3\beta_3\gamma\epsilon^*$ for distinction. The mass spectrum in figure S5a shows that the tags are not responsible for destabilization of the complex.

Remarkably the *in vivo* complexes are not only stable without ATP/Mg²⁺ addition which is crucial for the *in vitro* complexes, but even the last assembly step -association of δ to *in vivo* purified $\alpha_3\beta_3\gamma\epsilon$ and $\alpha_3\beta_3\gamma\epsilon^*$ pre-complexes forming the full F_1 -complex (Fig. S5) is ATP/Mg²⁺ independent.

The role of ATP hydrolysis vs. nucleotide binding for the *in vitro* assembly process of bacterial F_1 -ATPase

The important role of ATP hydrolysis for the function of ATP synthases is unquestioned and we have as well observed the significance of ATP for complex assembly. Nevertheless, it is not yet clear, whether ATP hydrolysis or only nucleotide binding is required for the F_1 assembly process. Therefore, we repeat the described assembly experiments with α , β and $\gamma\epsilon$, but replace ATP by non-hydrolysable ATP analogues, such as ADP, adenylyl-imidodiphosphate (AMP-PNP) and adenosine 5'-[γ -thio]triphosphate (ATP- γ -S) with a concentration of ([ATP/ATP-analogues]/[Mg²⁺] = 2 mM).

The obtained MS-spectra with non-hydrolysable ATP analogues (Fig. 4b-d) show the successful formation of larger F_1 -subcomplexes. Interestingly all ATP analogs allow formation of $\alpha_3\beta_3\gamma\epsilon$ apart from AMP-PNP, which generates only $\alpha_2\beta_2\gamma\epsilon$ subcomplexes and not the expected $\alpha_3\beta_3\gamma\epsilon$ complex; which could be due to differences in the chemical geometry of AMP-PNP. The free lone pair of nitrogen might sterically inhibit the assembly of the last $\alpha\beta$ heterodimer to the complete $\alpha_3\beta_3$ hexamer. Generally, this confirms that ATP hydrolysis is not required for assembly, but nucleotide binding is the essential factor triggering the *in vitro* complex formation.

Importance of charged residues in the catalytic sites of bacterial F-type ATP synthases.

After determining the crucial effect of ATP binding for ATPase assembly, we want to shed more light on this process. Recent studies have shown the importance of charged residues for the ability of P_i – binding at the catalytic sites of the $\alpha\beta$ interface of the hexameric head. The replacement of charged amino acids with neutral residues induced reduced cell growth and loss of ATPase activity, suggesting the electrostatic interactions between amino acids to be crucial for initial phosphate binding in the catalytic sites^[39]. Specifically four residues β (K155), β (R182), β (R246) and α (R376) were shown to be critical^[41–43]. We want to deduce if these mutations affect ATPase activity via their influence on the ability of the complex to bind/hydrolyze ATP or if the effect might be even more drastic and influence the complexes assembly. Therefore, we introduced homologous point mutations in conserved regions in α (R363Q, R363K) and β (K159Q, R186Q, R251Q) in the *A. woodii* operon (Fig. 5).

To investigate the effect of these charged residues in the catalytic site we first analyze the binding efficiency of ATP to α , β and different mutants with nano electrospray-ionization (nESI) (Fig. 6), which allows to observe the number of bound ATP. The deconvoluted MS-spectrum in Fig. 6a shows three peaks corresponding to β [WT] with zero, one or two bound ATP's. The dominating species is clearly the one with one bound ATP, indicating specific binding. In contrast peaks trailing of for the mutant β [K159Q] (Fig. 6b) suggest non-specific ATP binding only. Figure 6c shows the differences in ATP binding for the investigated α and β subunits and their respective mutants. The β [K159Q] mutant demonstrates a decrease of ATP binding around 60%, if compared to β [WT]. A less pronounced effect is seen for β [R251Q]. No significant differences in ATP binding were observed for α [WT] and its mutants which have a generally lower ATP-binding efficiency compared to β [WT].

Seeing that charged residues in the catalytic site can influence ATP-binding, we wanted to assess, if reduced ATP-binding affinity had an effect on the *in vitro* assembly process. Therefore, we performed the same HPLC and MS-experiments as for the wildtype α - β incubations. Figure 6d-h show with HPLC the formation of $\alpha\beta$ heterodimers at an elution peak time of 10.8 min for α [WT] with each β construct. Similarly, the *in vitro* assembly of β [WT] with α [R363Q] (Fig. 6i) or α [R363K] (Fig. S3a) show heterodimer formation. Interestingly, the HPLC runs for α [WT] with β [K159Q] or β [R251Q] (Fig. 6e/g) the mutants, which reduced ATP binding, show additional peaks at around 11.7 min suggesting the presence of unassembled monomers and therewith less efficient binding.

In vitro assembly of the α and β mutant constructs was then probed by LILBID-MS. Interestingly, we observe that under the same experimental conditions, which show stable heterodimers of wildtype α and β (Fig. 1b), we do not detect stable $\alpha\beta$ heterodimers for α [WT] incubated with β [K159Q] (Fig. 7a) or any of the b mutants (Fig. S4). This suggests that all b mutations affect stability to the extent that dimers can still be observed with HPLC (to a different degree) but are much reduced in the LILBID spectra. Similarly, we see clearly decreased dimerization for β [WT] with α [R363Q] (Fig 7c). A point mutation at the same position which retains the charge of the original amino acid (α [R363K]) shows no effect, as it does not hinder the formation of the $\alpha\beta$ heterodimer (Fig. S3).

As our experiments so far indicate that the formation of the $\alpha\beta$ heterodimer is decisive for further complex formation, the mutations, which impede the heterodimer, should affect the formation of higher order F_1 -complexes as well. Our mass spectra confirm that incubation of α [WT], β [K159Q] and the central stalk $\gamma\epsilon$ does not lead to the $\alpha_3\beta_3\gamma\epsilon$ complex (Fig. 7b), which we observed with the wildtype β (Fig. 3c). The same result is obtained with all other β mutants, (β [R186Q], β [R251Q] and β [K159Q, R186Q, R251Q], highlighting the important role of the charged residues in subunit β not only for the catalytic activity but as well for the F_1 assembly (Fig. S4). In contrast assembly of the mutant α [R363Q], β [WT] and the central stalk $\gamma\epsilon$ generates a larger complex – but interestingly not the expected hexameric head but subcomplex $\alpha_2\beta_2\gamma\epsilon$ (Fig. 7d). This indicates that replacing the arginine with the neutrally charged glutamine in subunit α has an effect on the assembly process, which is less pronounced than the effect of the above-mentioned substitutions in subunit β , but still hinders the formation of the correct $\alpha\beta$ hexamer.

Order of assembly steps into F_1

The combined results obtained with LILBID and nESI-MS and HPLC allow us to establish all subcomplexes which form *in vitro* en route to the full complex as well as the necessary assembly conditions. As these assemblies take place in minimal environment, compared to a cell, we need to guarantee that the observed subcomplexes are not just the result of aggregation, but can occur in the same manner in the cell. Therefore we expressed the subunit combinations, which we found in our assembly experiments directly in *E. coli* cells for comparison. We were able to obtain all anticipated subcomplexes with the same stoichiometries. Based on these findings we can follow the individual assembly steps and establish their order for formation of bacterial soluble F_1 domain of F-type ATP synthases (Fig. 8).

Overall our results show that, α - and β -subunits assemble specifically to $\alpha\beta$ heterodimers only in presence of nucleotides and Mg^{2+} (Fig. 1, Fig. 3 and Fig. 4) while surprisingly not forming higher subcomplexes (e.g. $\alpha\beta_2$, $\alpha_2\beta$, $\alpha_2\beta_2$ etc.) least of all the expected hexameric head ($\alpha_3\beta_3$). The α and β subunits can both bind individually to the central stalk γ , but only little binding is observed and in a maximal copy number of one (Fig. S8c-d), unless α and β dimerize first. Three pairs of $\alpha\beta$ heterodimers bind either to the central stalk γ or a preformed $\gamma\epsilon$ complex to generate stable $\alpha_3\beta_3\gamma$ or $\alpha_3\beta_3\gamma\epsilon$ complexes, respectively. The relevance of these *in vitro* results is shown in our heterologously expressed subcomplexes $\alpha_3\beta_3\gamma$ and

$\alpha_3\beta_3\gamma\epsilon$ which we can isolate as stable and bioactive complexes *in vivo* (Fig. S6). The δ -subunit binds to the *in vivo* or *in vitro* pre-complexes containing the hexameric head (Fig. 3d and Fig. S5), which generates the full F_1 complex.

As isolated subunit γ could not be purified in *E. coli* for stability reasons, we purified $\gamma\epsilon$ (Fig. S7), so our assembly studies did not include binding of ϵ and γ analogously to all the other binding experiments. The complete aggregation of the γ -subunit into inclusion bodies in the bacterial matrix during the overexpression, could suggest that the ϵ -subunit is essential for the stabilization of the central stalk (Fig. S7), which would place binding of ϵ to γ as a first step parallel to the formation of the $\alpha\beta$ heterodimer, before further assembly steps occur. Nevertheless, expression of *in vivo* subcomplexes showed that $\alpha_3\beta_3\gamma$ can be expressed as a stable subcomplex, which indicates that binding of ϵ is not an essential prerequisite for any further assembly to the F_1 complex. ϵ could therefore enter the assembly network at different times, as we indicate in Fig 8.

Interestingly, we observe some differences between our *in vivo* and *in vitro* complexes: the *in vivo* pre-formed complexes are stable without nucleotide- and Mg^{2+} and the binding of δ to any complexes including the hexameric head can then be performed *in vitro* without nucleotide- and Mg^{2+} addition. The same assembly experiment with the *in vitro* formed $\alpha_3\beta_3\gamma\epsilon$ complex requires nucleotide- and Mg^{2+} addition, to hinder disaggregation of the complexes. This could be explained, if the F_1 -subcomplexes formed *in vivo* and *in vitro* shared the same assembly pathway and stoichiometry, but a structural change occurring in the cell to stabilize the formed complex were missing *in vitro*, which is a question we will come back to later. Fig. 8 depicts a full summary of the different assembly steps of the soluble part F_1 as determined by our experimental data. The entire assembly process follows a choreographed pathway, which allows for an efficient construction of the F_1 complex.

ATPase activity of *in vitro* assembled *A. woodii* $\alpha\beta$ and $\alpha_3\beta_3\gamma\epsilon$ complex

In general, many macromolecular complexes need molecular chaperones, which assist for example in the conformational folding or assembly processes. In mitochondria, it is known that the F_1 - assembly of the hexamer of alternating α - and β -subunits requires two specific chaperones, Atp11p and Atp12p, that bind transiently to β and α ^[31,44]. Our previous results demonstrate that the bacterial F_1 - assembly can take place without the presence of chaperones. This was a surprising finding, as a generally accepted hypothesis expects an Atp12p homolog, which has been identified only in proteobacteria^[45] so far, to play an essential role in the bacterial F_1 - assembly. The question arises, if chaperones are not needed at all, or if their presence might be required, if not for assembly, then to guarantee correct function and activity.

For this reason, we investigated our *in vitro* assembled complexes for bioactivity. We performed ATP hydrolysis experiments with all of the separately expressed constructs ($\alpha\beta$, $\gamma\epsilon$ and ϵ) and the *in vitro* assembled $\alpha\beta$ and $\alpha_3\beta_3\gamma\epsilon$ complexes using an enzyme-coupled activity assay^[37,38]. For *in vitro* complex formation the subunits were incubated with 4 mM $MgCl_2$ and ATP with subunit stoichiometries of 1:1 for

the $\alpha\beta$ heterodimer, and 3:3:1 for $\alpha_3\beta_3\gamma\epsilon$. All subunits and subcomplexes showed moderate ATPase activity (Fig. 9a).

We could determine ATPase activities at 4 mM ATP for the β - and α -subunit of $0.15 \pm 0.05 \cdot 10^{-6}$ mol ATP / (mol protein $\cdot$ s) and $0.20 \pm 0.14 \cdot 10^{-6}$ mol ATP / (mol protein $\cdot$ s), respectively. These are quite similar and do not reflect the threefold higher ATPase activity of α previously seen in *E.coli*^[30].

As isolated subunit ϵ was already known to bind ATP^[46] and has to undergo large conformational changes to regulate the ATPase^[47] we hypothesized that ϵ as well undergoes hydrolysis. Interestingly, the isolated ϵ -subunit showed a hydrolytic activity at $3.4 \pm 0.1 \cdot 10^{-6}$ mol ATP / (mol protein $\cdot$ s), which is one magnitude higher than that of the single catalytic β -subunit. Enzymatic hydrolytic activity of $\gamma\epsilon$ was measured at $3.1 \pm 1.4 \cdot 10^{-6}$ mol ATP / (mol protein $\cdot$ s) placing the overall activity of $\gamma\epsilon$ very similar to ϵ , suggesting no contribution from γ .

The ATPase activity calculated for the *in vitro* assembled $\alpha\beta$ heterodimer is $0.69 \pm 0.22 \cdot 10^{-6}$ mol ATP / (mol protein $\cdot$ s), assuming incubation leads to full complex formation. As this might not be the case, all molar activities of *in vitro* complexes have to be seen as a conservative estimate – if full complexation could be guaranteed the observed values would likely be higher. Nevertheless, the observed hydrolytic activity is about twice the sum of the individual α and β activities, supporting the relevance of the $\alpha\beta$ heterodimer as a building block and working unit of the ATPase. Surprisingly this value stays clearly below the hydrolytic activity of ϵ .

The reconstitution based on the incubation of α , β and $\gamma\epsilon$ resulted in a hydrolytic enzymatic activity of $6.1 \pm 0.5 \cdot 10^{-6}$ mol ATP / (mol protein $\cdot$ s). Interestingly, the activity of this *in vitro* assembly is not much higher than the sum of the ATPase activities of the utilized subunits. The increased activity (factor 1.5) of the of the now complexed subunits stems mainly from the increase of activity seen for the formation of the three required $\alpha\beta$ heterodimers.

Similar measurements were performed with *in vivo* purified $\alpha\beta$ and $\alpha_3\beta_3\gamma\epsilon^*$. The ATPase activity for both is noticeably beyond anything we observed for *in vitro* complexes. ($160 \pm 14 \cdot 10^{-6}$ and $900 \pm 7 \cdot 10^{-6}$ mol ATP / (mol protein $\cdot$ s) respectively) (Fig. 9a and 9b) These values cannot be compared directly with those obtained for *in vitro* complexes, for which the individual subunits don't fully form into complexes. Nevertheless, the activity of the *in vivo* $\alpha\beta$ heterodimer alone is already much higher than that of the *in vitro* assembled $\alpha_3\beta_3\gamma\epsilon$ complex. Similarly, the *in vivo* $\alpha_3\beta_3\gamma\epsilon^*$ shows an activity way beyond the sum of its components. This indicates that the *in vitro* conditions allow for the correct assembly of the functional subunits, which then retain their individual ATPase activity, while the cellular environment allows for modifications, which increase the ATPase activity of the $\alpha\beta$ heterodimer and the whole ATPase, way beyond that of the sum of its parts.

Stability studies with ion mobility MS: *in vivo* vs *in vitro* heterodimers

The observed differences in bioactivities as well as stability without ATP/Mg⁺ for our *in vivo* and *in vitro* complexes suggest that additional effects besides assembly are required to form an active complex. We assume conformational changes to play a role, possibly triggered by chaperones.

The relevance of the $\alpha\beta$ heterodimer formed as a first step in the ATPase assembly and the differences observed in our *in vivo* and *in vitro* studies, makes the heterodimer a very interesting candidate to investigate, if the changes in activity can be correlated to structural changes. Noticeable structural rearrangements should be accompanied by a change in collision cross section (CCS), which can be monitored by ion mobility (IM) MS, while changes to the intrinsic stability can be revealed by differences in collision induced unfolding (CIU) experiments. To validate our assumption, we investigate the heterodimer for differences between *in vivo* and *in vitro* formed complex. We select in both cases the 20-times charged $\alpha\beta$ heterodimer in our nESI mass spectra as precursor ion which is analyzed via ion mobility MS in dependence of the applied collision voltage. IM allows the separation of ions based on their mobility through an inert gas under the influence of an electric field. Increasing the collision voltage can lead to collision induced unfolding and then dissociation of the complex. The energy required to cause CIU can be correlated to the complex stability. In the resulting IM fingerprint of the *in vivo* assembled $\alpha\beta$ heterodimer, the IM signal appears for low collision voltages at drift times about 17.5 ms (Fig. 10), which corresponds to the compact feature of the folded complex. Increase of collision voltage to around 175 V leads to protein unfolding, which is accompanied by an increase in CCS. The original IM signal decreases partially in favor of a signal at 19 ms, representing the corresponding unfolded structure of the $\alpha\beta$ heterodimer. In contrast the *in vitro* assembled $\alpha\beta$ heterodimer already unfolds at collision voltages about 155 V and appears at a slightly higher drift time (about 20 ms) compared to the *in vivo* assembled $\alpha\beta$. This indicates that the *in vitro* structure is less stabilized against unfolding than the *in vivo* assembled $\alpha\beta$. This can be seen more clearly in the difference plot (*in vitro* minus *in vivo*, Fig 10c), which helps to compare specific features of each fingerprint directly. Additionally it reveals a significantly broader IM signal for the compact, as well as the unfolded state for the *in vitro* assembled $\alpha\beta$ heterodimer indicating a less homogenous structure for the *in vitro* than for the *in vivo* $\alpha\beta$ heterodimer. A more defined structure and increased stability of the *in vivo* heterodimer are structural properties, which can be correlated to the increased ATPase activity.

Discussion

The aim of this study is better understanding of the assembly process of an ATPase – a large heterogeneous macromolecular complex, with eight different subunits. We determined conditions required for the whole *in vitro* assembly process into the soluble part F₁ of *A. woodii* ATPase from single purified F₁-subunits. We followed the assembly process *in vitro* step by step from subunits via the subcomplexes, which form (or don't form) after incubation of different subunit combinations. To verify that the observed complexes on pathway to the F₁ complexes are meaningful and can assemble in the same stoichiometries in the cell, we expressed a variety of *in vivo* F₁ subcomplexes. We could purify all of the on-pathway complexes *in vivo*, which allowed comparison of *in vitro* vs. *in vivo* formed complexes.

While for the formation of an $\alpha_3\beta_3$ hexamer has been shown to occur even independently of nucleotide or Mg^{2+} [48], we find a different situation here for the mesophilic *A. Woodii* where the presence of ATP/ Mg^{2+} is essential for complex formation (Fig. 1, Fig. 3), up to the $\alpha_3\beta_3\gamma\epsilon$. We reveal that it is ATP binding which triggers the *in vitro* assembly and not ATP-hydrolysis (Fig. 4).

We could demonstrate that the incubation of α and β leads to formation of the heterodimer without formation of any higher $\alpha\beta$ oligomers. Only incubation in the presence of the central stalk $\gamma\epsilon$ allowed further assembly into larger complexes including the hexameric head on pathway to the F_1 (Fig. 2). The lack of complexes with odd numbers of α and β subunits (Fig. 3, Fig. 4, Fig. S6) shows that the formation takes place with the $\alpha\beta$ heterodimers as basic building blocks. As we were able to express and purify intact $\alpha_3\beta_3\gamma$ complexes without the presence of ϵ we can conclude that it is the central stalk subunit γ , which is crucial for generating a stable $\alpha_3\beta_3$ hexagon out of $\alpha\beta$ heterodimers, while ϵ can join at different stages of the assembly. Including δ in our assembly studies could show that δ does not bind to isolated α or β (Fig. S8a-b). Only after formation of the hexameric head, the subunit δ can bind, resulting in the full F_1 complex (Fig. 2, Fig. S5), which can occur ATP/ Mg^+ independent (Fig. S5). Overall, our *in vitro* assembly experiments in combination with the complexes which can be assembled *in vivo* give a full picture of a well-choreographed assembly process, as shown in Figure 8.

Point mutations in the catalytic interface of α (at position R363), and β (K155, R182, R246) which replace positively charged amino acids with neutral residues, demonstrated that such modifications can but don't have to have a direct effect on ATP binding efficiency. HPLC runs still showed the *in vitro* assembly of $\alpha\beta$ heterodimers for all combinations of one wildtype and one mutated subunit (Fig. 6d-i), albeit with affected complex stability, as incomplete dimer assembly is observed for those mutants with reduced ability to bind ATP. This effect on stability is even more pronounced in the LILBID-MS results, which show reduced or no $\alpha\beta$ dimer for all mutants which substitute a charged with a neutral amino acid (Fig 7a and 7b, Fig. S4). Substitution of one charged amino acid for another (α R363K) had no influence. This demonstrates that those point mutations, which remove a charge in the $\alpha\beta$ interface, cause a decrease of $\alpha\beta$ heterodimer stability. *In vitro* assembly experiments with α and any β mutant, which included $\gamma\epsilon$ did not yield any higher complexes. An interesting finding was that for the α mutant (α R363Q), stable $\alpha\beta$ heterodimers and even higher F_1 subcomplexes, including one or two heterodimers (Fig. 7c and Fig. S3) can still form, albeit no complex with a complete hexameric head can be detected (Fig. 7d). A similar disturbance was found for incubations of wildtype subunits with AMP-PNP, which might sterically influence the assembly. This shows that even if $\alpha\beta$ dimer formation is possible, the assembly of the hexameric head, which has to function via large conformational changes in a very tightly controlled manner, is a precise process, which can be hindered by small disruptions.

Our *in vitro* assembly studies prove that no direct involvement of chaperones is necessary for the correct reconstitution of the F_1 complex from the individual subunits. Based on the known relevance of chaperones for complex formation in cells this is a surprising finding. Therefore, the question arises: If chaperones are not essential for the *in vitro* assembly of the F_1 -ATPase, could they be relevant to ensure

the bioactivity and function of the enzyme? To shed light on this question, we correlate our assembly results with an enzyme-coupled activity assay for single isolated F_1 -subunits, *in vivo* and *in vitro* formed complexes (Fig. 9). The first building block is the $\alpha\beta$ heterodimer. The *in vitro* assembled $\alpha\beta$ shows an activity, which is about twice as high than the mere sum of the α and β activity. This effect is not big, so could be based on the ATP hydrolysis taking place in the $\alpha\beta$ interface, which would offer a better binding pocket for the nucleotide. A much larger effect is seen in the comparison with the *in vivo* $\alpha\beta$ dimer. Mass spectrometric analysis (Fig. 1b) and HPLC (Fig. 1c) measurements confirm the efficient, albeit not complete heterodimer assembly, so exact comparison of *in vivo* and *in vitro* activity is difficult. Should about half of the possible dimer form *in vitro* a conservative estimate would put the activity of the *in vivo* purified protein complex to be at least a factor of 100 above that of the *vitro* assembled complex. This suggests a principle difference in the heterodimers, leading to a much higher bioactivity of complexes formed in a cell. This could be explained by a structural difference of the *in vitro* and the *in vivo* formed heterodimer.

To further investigate this assumption we perform stability studies with *in vivo* (Fig. 10a,d) and *in vitro* (Fig. 10b,e), formed $\alpha\beta$ heterodimers by means of mass spectrometry CIU experiments, which we monitor by ion mobility MS. At low collision voltages the IM plots show signal at the same drift time (around 17.5 ms) for both complexes. Noticeable is the comparably broad signal distribution for the *in vitro* complex as opposed to the *in vivo* dimers. Comparison of both IM plots is most intuitive in the difference spectrum of both IM plots (Fig. 10c,f). This implies that the *in vivo* assembled $\alpha\beta$ heterodimer is present with a more homogenous structure, while the *in vitro* formed complexes seem less defined. The comparison of both complexes during the CIU experiment demonstrates that *in vitro* assembled $\alpha\beta$ unfolds at a lower voltage than the *in vivo* formed heterodimer, validating that the *in vivo* formed complex is intrinsically more stable than the *in vitro* construct. These are interesting observations in the light of the subunit β 's ability to undergo four different conformations with different nucleotide binding affinities, tight (T), loose (L), closed (C) and open (O) resulting in ATP hydrolysis/synthesis^[49]. The stable and homogeneous conformation found for the *in vivo* complex might suggest a potentially chaperone induced bias towards a single conformation, which supports assembly. In our *in vitro* assembly assays no such bias towards a specific conformation is induced, explaining the differences in stability and hydrolysis activity. This structural difference propagates to the whole *in vitro* F_1 which can be seen in the dependence on ATP/Mg⁺ of the *in vitro* F_1 complexes to prevent dissociation (Fig. S2e and S2f), which is less of an issue for their *in vivo* counterparts (Fig. S6). This will as well be the reason behind the much higher ATPase activity of the *in vivo* complexes. A precise investigation of the structural conformation of the subunits/subcomplexes compared to *in vivo* complexes will be the focus of future studies.

What is known about the role of the ϵ subunit so far, is its relevance in coupling of the soluble part F_1 with the membrane-embedded F_0 and its regulatory role in the inhibition of the ATP hydrolysis activity^[17,20,21]. Interestingly, our ATP activity assays revealed that isolated purified ϵ itself shows an ATP hydrolysis activity which is surprisingly even higher than for subunit β . The ϵ -subunit in bacterial F_1F_0 is known to perform its regulatory function, by taking a hairpin or extended conformation,^[7,50] and isolated

subunit ϵ of *Bacillus subtilis* was found to be able to bind ATP^[46]. Our results suggest that this extensive conformational change is driven by ATP hydrolysis.

All in all we could show that the soluble F_1 part can be reconstituted *in vitro* from the purified F_1 -subunits in presence of nucleotide and Mg^{2+} . Our studies have provided an exciting overview of the step by step assembly via well-defined subcomplexes towards the full complex, with a preformed $\alpha\beta$ heterodimer as essential building block. Our results show the special roles especially of subunits β and ϵ for the functional complex. Differences in the ATPase activity of the generated F_1 complex generated *in vitro* and *in vivo* seem to be based on structural differences suggesting that bacterial chaperones may not be needed for correct stoichiometric assembly of the ATPase but assist to fine-tune the assembly into a fully functional F_1 .

Declarations

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Figures

Figure 1

Dependence of ATP/Mg²⁺ in the assembly process of bacterial F-type ATP synthases. (a) LILBID spectrum of in vitro α and β assembly: The α - (light blue) and β -subunit (dark blue) either without (a) or with (b) 2 mM ATP and 2 mM MgCl₂. In presence of ATP/Mg²⁺ the mass spectrum shows the formation of a specific $\alpha\beta$ heterodimer assembly, but no higher $\alpha\beta$ oligomers. (c) High Performance Liquid Chromatography (HPLC) of in vitro α and β assembly: The addition of 2 mM ATP and 2 mM MgCl₂ to α and β lead to $\alpha\beta$ heterodimer formation displayed by a shift from 12.27 minutes (light green area) to approximately 11.26 minutes (light red area). The elution peak time of 13.25 minutes at 750 mAU is due of the high ATP concentration. (d) Model of bacterial F-type-ATPase: The soluble module F₁ is organized by α - (light blue), β - (dark blue), γ - (dark green), δ - (orange) and ϵ -subunit (light green).

Figure 2

(a) Summary of all in vitro assembled subcomplexes observed in LILBID-MS: Assembly variations of isolated subunits α , β , δ and ϵ . (b) SDS-PAGE (NuPAGE 4-12% Bis-TRIS) of single purified subunits α , β ,

$\gamma\epsilon$ and δ . Affinity purification of α with Strep-Tactin- and β , $\gamma\epsilon$ and δ with Ni-chelating chromatography. (c) List of in *E. coli* heterologously purified F1 and subcomplexes. All complexes were analyzed with LILBID-MS and SDS-PAGE (Fig. S6).

Figure 3

ATP/Mg²⁺ dependence of the in vitro assembly process of the full F1 complex of Na⁺-F1FO- ATP synthases of *A. woodii*: The α - (light blue), β -subunit (dark blue), $\gamma\epsilon$ -complex (dark and light green) were incubated at 4°C for 1 hour either without or with 2 mM ATP and 2 mM MgCl₂ (spectra on the left). Then the δ -subunit (light yellow) was added (Spectra on the right). (a- b) No specific assemblies into higher F1-subcomplexes could be detected without ATP/Mg²⁺. (c-d) In presence of ATP/Mg²⁺ the fully assembled F1 can be identified with additional F1 subcomplexes (e.g. $\alpha_2\beta_2\gamma\epsilon$, $\alpha\beta\gamma\epsilon$, $\alpha\beta\gamma$) implying pairwise specific $\alpha\beta$ heterodimer association. The δ -subunit can only bind when a complex containing the hexameric head domain $\alpha_3\beta_3$ is already pre-formed.

Figure 4

Role of ATP hydrolysis vs. nucleotide – binding in the assembly process to F1 of bacterial F-type ATP synthases. LILBID spectra of in vitro α , β , $\gamma\epsilon$ assembly: The α - (light blue), β -subunit (dark blue), $\gamma\epsilon$ -complex (dark and light green) were incubated with 2mM MgCl₂ and 2mM of a nucleotide at 4°C for 1 hour. Complex formation is observed after incubation with (a) ATP (b) AMP-PNP (c) ADP. (d) ATP- γ -S. Full $\alpha\beta\gamma\epsilon$ assembly can be identified next to small amounts of other F1 subcomplexes (e.g. $\alpha\beta\delta\gamma\epsilon$ $\alpha\beta\delta\gamma\epsilon$, $\alpha\beta\gamma\epsilon$, $\alpha\beta\gamma$), for ATP as well as the non-hydrolysable ATP-analogues, apart from AMP-PMP, where the highest observed complex is $\alpha\beta\delta\gamma\epsilon$.

Figure 5

Amino acid sequence alignment of α - and β -subunit residues. (a) Escherichia coli (*E. coli*) and (b) Acetobacterium woodii (*A. woodii*). Positions of positively charged amino acids which were mutated are marked in blue.

Figure 6

Dependence of ATP-binding of α , β and their mutants and the formation of $\alpha\beta$ heterodimers. (a-b) ATP-binding: Deconvoluted nESI-MS-spectra of ATP-binding of β [WT] and β [K159Q]. (c) Summary of ATP-binding of β [K159Q], β [R186Q], β [R251Q], β [K159Q, R186Q, R251Q], α [WT], α [R363Q] and α [R363K] relative to β [WT]. (d-i) HPLC measurements: In vitro $\alpha\beta$ heterodimer assembly of α [WT] with β [WT] and β mutants.

(j) HPLC measurement: In vitro $\alpha\beta$ heterodimer assembly of β [WT] with α [R363Q]. Data were collected in biological replicates ($n = 3$).

Figure 7

Role of charged residues in the catalytic site for the assembly process analyzed by LILBID-MS. (a) In vitro assembly of α [WT] with β [K159Q] mutant shows no significant formation of the $\alpha\beta$ heterodimer, which we observe for the α [WT] and β [WT] (Fig. 1b). (b) The addition of the central stalk $\gamma\epsilon$ does not lead to the formation of the desired $\alpha3\beta3\gamma\epsilon$ complex. (c) In vitro assembled α [R363K] and β [WT] revealed reduced $\alpha\beta$ heterodimer assembly. (d) Addition of $\gamma\epsilon$ leads to formation of maximally a $\alpha2\beta2\gamma\epsilon$ subcomplex with charge states (-1 to -4).

Figure 8

Order of assembly steps into bacterial F1. (a) As a first step α -subunits (light blue) and β -subunits (dark blue) form $\alpha\beta$ heterodimers, which requires the presence of nucleotides (NT) and Mg^{2+} . (b) As next step three of these heterodimer building blocks assemble pairwise to the central stalk γ (dark green). Only after the formation of the full hexameric head, the δ -subunit (yellow) is able to bind. The ϵ -subunit (light green) can join into the in the assembly process at all intermediate steps en route to the F1 complex.

Figure 9

ATP hydrolysis of (a) isolated α , β , ϵ , $\gamma\epsilon$ and in vitro assembled $\alpha\beta$ and $\alpha\beta\gamma\epsilon$ complexes, as well as (b) in vivo $\alpha\beta$ and $\alpha\beta\gamma\epsilon^*$ complexes. (b) Spectroscopic comparison of the decrease in absorbance at 340 nm per time [s] in the enzyme-coupled ATPase activity assay of the in vitro assembled $\alpha\beta$ from isolated α and β (5.5 μ M each) relative to the in vivo produced $\alpha\beta$ (0.5 μ M protein complex) in 100 μ L (100 mM TRIS, 100 mM maleic acid, 5 mM $MgCl_2$, 3 mM phosphoenolpyruvate (PEP), 4 mM ATP, 0.5 mM NADH, 10 units L-lactate dehydrogenase (L-LDH), 10 units pyruvate kinase (PK), pH 7.5).

Figure 10

CIU fingerprints of the in vivo (a) and in vitro (b) assembled $\alpha\beta$ heterodimer. At low collision voltages the IM signal of the $\alpha\beta$ heterodimer appears in both cases at a drift time about 17.5 ms. At higher collision voltages about 175 V the signal of the in vivo assembled $\alpha\beta$ heterodimer starts to unfold, resulting in a signal at about 19 ms. In contrast, the in vitro formed $\alpha\beta$ heterodimer already unfolds at lower collision voltages about 155 V. (c) The difference plot of both fingerprints illustrates the change regarding unfolding behavior between in vitro and in vivo assembled heterodimers. (d – e) show the drift times for different collision voltages for both cases and the difference plot (f). The overall feature of the in vitro $\alpha\beta$ is clearly broader than for the in vivo $\alpha\beta$.

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