Proton transport coupled ATP synthesis by the purified yeast H⁺-ATP synthase in proteoliposomes

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The H⁺/ATP synthase from yeast mitochondria, MF₀F₁, was purified and reconstituted into liposomes prepared from phosphatidylcholine and phosphatidic acid. Analysis by mass spectrometry revealed the presence of all subunits of the yeast enzyme with the exception of the K-subunit. The MF₀F₁ liposomes were energized by acid–base transitions (ΔpH) and a K⁺/valinomycin diffusion potential (Δφ). ATP synthesis was completely abolished by the addition of uncouplers as well as by the inhibitor oligomycin. The rate of ATP synthesis was optimized as a function of various parameters and reached a maximum value (turnover number) of 120 s⁻¹ at a transmembrane pH difference of 3.2 units (at pHₘ₈ = 4.8 and pHₘᵦ₈ = 8.0) and a Δφ of 133 mV (Nernst potential). Functional studies showed that the monomeric MF₀F₁ was fully active in ATP synthesis. The turnover increased in a sigmoidal way with increasing internal and decreasing external proton concentration. The dependence of the turnover on the phosphate concentration and the dependence of Kₐ₅ on pHₘ₈ indicated that the substrate for ATP synthesis is the monoanionic phosphate species H₂PO₄⁻.

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1. Introduction

Membrane-bound H⁺-ATP synthases (H⁺-translocating adenosine triphosphatase EC 3.6.1.14) synthesize ATP from ADP and inorganic phosphate using the energy of a transmembrane electrochemical potential difference of protons [1–3]. They occur in the plasma membranes of bacteria, in the thylakoid membrane of chloroplasts and in the inner membrane of mitochondria [4–8]. H⁺-ATP synthases consist of a hydrophilic F₁-part (subunits αβγδε) containing the nucleotide and Pₐ binding sites and of a hydrophobic membrane integrated F₀-part containing the proton binding sites (subunits αβ₆ε₁₀–₁₂) (subunit composition and nomenclature of E. coli). The kinetics of the enzyme is described by the binding change theory which explains the cooperativity of the three catalytic sites by rotation of the γ-subunit within the αβ₆ε₁₀–₁₂-barrel [9,10]. The first high resolution structure of the F₁-part, from bovine heart mitochondria, corroborated this theory [11]. A crystal structure of the holo-enzyme (F₀F₁) was obtained for the yeast mitochondrial enzyme [12], which revealed a ring structure with 10 c-subunits and their interaction with the γ-subunit. Anti-clockwise (viewed from the membrane side) rotation of the γ-subunit during ATP hydrolysis was observed in bacterial F₁-parts [13]. Movements of the γ- and ε-subunit relative to the stator subunits during ATP synthesis and opposite movements during ATP hydrolysis have been shown by single pair fluorescence spectroscopy with membrane integrated EF₀F₁ [14,15].

To investigate the mechanism of coupling between proton transport and ATP synthesis H⁺-ATP synthases from bacteria and chloroplasts have been isolated, purified and reconstituted into liposomes. In these reconstituted systems high rates of ATP synthesis (up to 200 s⁻¹) in response to ΔpH and Δφ generated in acid–base transitions have been reported – see e.g. [16–21]. A tremendous amount of biochemical, structural and functional work has been carried out with the F₁-part of mitochondrial H⁺-ATP synthases (MF₀F₁). However, much less is known about the coupling between proton transport and ATP synthesis. ATP synthesis by bovine sub-mitochondrial particles (SMP) driven by acid–base transition was reported [22], and from that work a turnover number in the order of 50 s⁻¹ can be calculated under the assumption that the H⁺-ATP synthase represents 10% of the total protein. Several isolation procedures of mitochondrial MF₀F₁ have been reported and after reconstitution into liposomes functional studies reveal that the...
enzyme appeared to be able to hydrolyze ATP, to energize the membrane by proton transport, and to catalyze ATP-Pi exchange — see e.g. [23–26]. Detectable ATP synthesis rates were reported, when MF0F1 was reconstituted with bacteriorhodopsin with turnover numbers ranging between $10^{-4}$ and $10^{-3}$ s$^{-1}$ [23,27,28]. These results contrast with the maximal turnover number of 440 s$^{-1}$ previously estimated in submitochondrial particles [29]. The reason for the low rates could well have been the low levels of protonotive force achievable with reconstituted bacteriorhodopsin [30]. High proton motive forces can be established by acid–base transitions. However, such studies with reconstituted MF0F1 have not been reported yet.

To obtain high ATP synthesis activities several problems must be solved: 1) The mitochondrial H$^\text{+}$-ATP synthase is more complex than the corresponding bacterial and chloroplast enzymes. It has 20 different subunits and it is possible that an important subunit is lost either during isolation or during the reconstitution procedure. 2) MF0F1 is able to form supramolecular complexes [31–33] and electron microscopy studies, both of detergent-solubilised MF0F1 complexes and of mitochondrial membranes revealed an angled arrangement of the monomers in dimers, and a ribbon-like organization in higher order oligomers [34–36]. MF0F1 oligomerization imposes a curvature on the inner mitochondrial membrane, and the resulting invaginations have been proposed to act as proton traps improving the efficiency of ATP synthesis [36,37]. However, the functional significance of MF0F1 oligomerization is not yet fully understood, in particular it is not known whether the monomeric form is capable of high activities. 3) The purified MF0F1 detergent micelle must be reconstituted into liposomes in a functionally active form. 4) The optimal conditions for measuring high activities must be established.

In this work MF0F1 was isolated, the subunit composition was determined by mass spectrometry and the monomeric enzyme was reconstituted into liposomes. Acid–base driven ATP synthesis by such monomeric enzyme resulted in rates as high as 120 s$^{-1}$. Moreover, it is shown that the substrate for ATP synthesis is the monoanionic species $H_2PO_4^\text{−}$.

2. Materials and methods

2.1. Cell growth and MF0F1 purification

Saccharomyces cerevisiae cells of the strain YRD15 (MATα his3-11,13-15 leu2-3,112, ura3-1,251,3-373[ρ+]) were grown in well-aerated, SACC$\text{−}$media [1% (w/v) yeast extract, 0.12% (w/v) (NH$_4$)$_2$SO$_4$, 0.1% (w/v) KH$_2$PO$_4$, 0.01% (w/v) CaCl$_2$, 0.0005% (w/v) FeCl$_3$, 0.07% (w/v) MgCl$_2$, 0.05% (w/v) NaCl, 2% (w/v) ethanol] supplemented with 20 mg/l of each leucine, histidine and uracil and stored at −80 °C. A total of 100 g cells was thawed in 200 ml of buffer A1 (10 mM Tris/HCl pH 7.0, 250 mM sucrose, 5 mM 6-aminohexanoic acid) and phenylmethylsulfonyl fluoride was added to a final concentration of 0.0025% (w/v). MF0F1 was isolated similarly as described in [12]. Cells were disrupted with glass beads at 4 °C as previously described [24] and the cell debris was removed by centrifugation at 10000 g for 30 min in a Beckman JA-16.250 rotor at 4 °C. Crude mitochondria were isolated by centrifugation of the supernatant at 23000 g for 30 min in the same rotor at 4 °C. The pellet was resuspended in 40 ml buffer B1 (20 mM HEPES, 250 mM sucrose, 5 mM 6-aminohexanoic acid, 40 mM NaCl, 4 mM MgCl$_2$, and 1 mM EDTA, titrated to a pH of 7.65 with NaOH). Submitochondrial particles (SMP) were prepared by sonicating the mitochondria for 3 min in a Branson Sonifier 250 (Output level 2, Duty Cycle 40%). Large particles were removed by centrifugation for 20 min at 4000 g in a Beckman JA-20 rotor at 4 °C. The SMP were collected by centrifugation for 30 min at 100000 g in a Beckman 60Ti rotor at 4 °C. The pellet was homogenized in 75 ml buffer B1 containing 0.0025% phenylmethylsulfonyl fluoride and 1.4% dodecylmaltoside and stirred for 40 min at room temperature. After addition of 75 ml cold B1, insoluble material was removed by centrifugation (30 min, 180000 g in a Beckman 60Ti rotor). The supernatant was applied to a 16 ml HQ20 column equilibrated with buffer C1 (20 mM HEPES, 250 mM sucrose, 1 mM EDTA, 4 mM MgCl$_2$, 5 mM 6-aminohexanoic acid, 1 mM dithiothreitol, 100 mM NaCl, 0.05% DDM, titrated to a pH of 7.65 with NaOH). The protein was eluted in a step gradient by increasing the NaCl concentration in buffer C1 from 100 mM NaCl to 184 mM NaCl. Fractions containing protein (determined photometrically) were pooled (approx. 20 ml) and concentrated in an Amicon Ultra-15 centrifugal filter (molecular weight limit 10 kDa). Gel filtration of the concentrated enzyme was carried out in buffer C1 with a 16/90 Sephacryl 300 column. Fractions containing MF0F1 were pooled (20 ml), concentrated as described above to a protein concentration of 5–10 μM, rapidly frozen and stored in liquid nitrogen. Total yield was approximately 10 mg. The concentration of MF0F1 was measured spectrophotometrically using the absorption coefficient at 280 nm calculated according to [39]. The absorption coefficients of the subunits are shown in Supplementary Table S1. The subunit composition of our MF0F1 preparation was determined by HPLC-electrospray mass spectrometry and, using the subunit stoichiometry reported in [40] ($\alpha$ββγβγδ 4αδβγδ) as shown in Supplementary Table S1 for nomenclature. In the following we used the absorption coefficient $ε_{280}=279$ 130 M$^{-1}$ cm$^{-1}$ for all concentration measurements. We assumed that all MF0F1 added to the reconstitution mixture was correctly incorporated into the membrane, which implies that the enzyme activities given in this work are the minimal activities.

2.2. Detection of monomeric and dimeric MF0F1 by Blue Native PAGE

SDS-polyacrylamide gel electrophoresis (SDS-PAGE) was carried out as described in [41]. The protein sample was incubated in 20 mM Tris pH 8, 2% (w/v) SDS, 1 mM DTT, 10% (v/v) glycerol and 0.04% bromophenol blue for 10 min at 95 °C and applied to a discontinuous acrylamide gel (13% gel overlaid with 4% sample gel).

Monomeric and dimeric MF0F1 were separated with Blue Native Polyacrylamide Gel Electrophoresis (BN-PAGE) as described previously [31]. MF0F1 was solubilized with Triton X-100 to protein ratios between 0.6 and 1.2 g/g protein from the isolated mitochondrial membranes. The solubilised proteins were applied to an acrylamide gradient gel (linear 4–13% gradient, overlaid with 4% sample gel).

2.3. Protein identification by HPLC–electrospray mass spectrometry

Lanes of a SDS-PAGE (13%) were cut into 38 horizontal 1 mm slices. Each slice was processed individually for establishing abundance profiles of identified peptides. Proteins were modified by iodoacetamide and in-gel digested as described [42]. Peptide mixtures were separated for nano-LC–ESI–MS/MS using a FAMOS Autosampler (Dionex), an Ultimate inert HPLC (Dionex) and an Agilent HPLC 1100 pump connected to the nano-ESI-Source of a Finnigan LTQ-FIT Thermo Electron) for online mass detection. Peptides were first collected on a trap column (0.1×15 mm, Zorbax Eclipse XDB-C18, 5 μm, Agilent Technology) for desalting and concentrating followed by separation on an analytical column made up by a fused silica emitter (0.075×150 mm, 6 μm, Proxeon Biosystems) filled with Pro C18, 3 μm (YMC). Peptides were eluted using a linear gradient from 97% water, 3% acetonitrile and 0.1% formic acid to 80% acetonitrile, 20% water and 0.1% formic acid within 60 min at a flow rate of 0.15 μl min$^{-1}$. Mass spectrometric detection consisted of full scans at a resolution of 25000 followed by data dependent selected ion scans at a resolution of 50000 and low resolution MS/MS scans using a dynamic exclusion of parent ion masses for 60 s.

The MS and MS/MS spectra were searched against Saccharomyces cerevisiae protein sequences deposited at the Uniprot database (release Feb 10, 2009) using an in-house installation of the program OMSSA (version 2.1) [43] as described [44]. Peptide hits were considered
significant if the precursor and product ion masses matched within 2 ppm and 0.5 rel. mass units, respectively, and if the E-value was below 0.01. Only the best hit per spectrum was considered. These criteria resulted in a peptide false positive rate of 0.1%. The peptide score was computed from the P-value of the program OMSSA as $-\log_{10}(P)$. Protein intensities were calculated as the sum of peptide integrated ion currents using the MSQuant program version 1.5 [45] and were used to compute protein distribution profiles [46]. For low intensity peptides which were not selected for data dependent MS/MS scans ion currents were manually integrated at a given mass over charge ratio using the Xcalibur Software (Thermo Electron). The latter method was used for subunit g.

2.4. Reconstitution of MF$_{0}$F$_{1}$ into liposomes

The reconstitution of MF$_{0}$F$_{1}$ into liposomes was carried out similarly as described in [47]. Preformed liposomes from phosphatidylcholine and phosphatidic acid (mass ratio 9:1) were prepared by sonication (3 × 30 s in a Branson Sonifier 250, Output level 4, Duty Cycle 90%) and by dialysis against buffer D1 (10 mM Tricine, 0.2 mM EDTA, 2.5 mM MgCl$_{2}$ and 0.25 mM diithiothreitol, titrated to pH 8.0 with NaOH). The lipid concentration after dialysis was 16 g/l. The liposomes were rapidly frozen and stored in liquid nitrogen. The size distribution of the liposomes was determined by photon correlation spectroscopy using a Zetamaster 9° ZEN 5002, Malver Instruments. For reconstitution of MF$_{0}$F$_{1}$, 150 μl liposomes were thawed and MF$_{0}$F$_{1}$ (6 μl, 7.5 μM in buffer C1), MgCl$_{2}$ (final concentration 2.5 mM), Triton X-100 (24 μl, final concentration 0.8% (w/v)) and 119 μl buffer E1 (20 mM succinate, 20 mM Tricine, 60 mM NaCl, 0.6 mM KCl, titrated to pH 8.0 with NaOH) were added. The reconstitution mixture was stirred slowly at room temperature for 1 h. Addition of Biobeads (35 mg per 100 μl of the protein/lipid/detergent solution) led to the removal of Triton X-100 and the insertion of MF$_{0}$F$_{1}$ into the lipid membrane [48]. In the end, the lipid concentration of the proteoliposomes was approximately 8 g/l with a MF$_{0}$F$_{1}$ concentration of 150 nM.

2.5. Measurement of ATP synthesis

The rate of ATP synthesis was measured at 25 °C similar as described earlier [17]. The proteoliposomes were energized by an acid–base transition and an additional K$^+$/valinomycin diffusion potential. The ATP concentration was monitored continuously with luciferin/luciferase (Roche) in a luminometer (LKB 1250). In order to generate the ΔpH, the proteoliposomes were incubated in the acidic medium [F1: 20 mM succinate, 0.6 mM KOH, 4 mM MgCl$_{2}$, 0.1–15 mM NaH$_{2}$PO$_{4}$, 0.4 mM ADP, 20 μM valinomycin (freshly added)]. To generate different ΔpH during the acid–base transition the acicd medium was titrated with NaOH to pH values between 4.7 and 6.7. The pH of the basic medium was constant: 250 mM Tricine, 120 mM KOH, 0.1–15 mM NaH$_{2}$PO$_{4}$, 4 mM MgCl$_{2}$, 0.4 mM ADP, titrated to pH 8.0 with NaOH.

ATP synthesis and detection of ATP with the luciferin/luciferase assay were carried out simultaneously as follows: 880 μl of the basic medium were mixed with 20 μl luciferin/luciferase reagent, placed in the luminometer and the base line was recorded. Proteoliposomes (15 μl, MF$_{0}$F$_{1}$ concentration 150 nM) were mixed with 100 μl acidic medium. The incubation time was varied between 2 min at pH$_{in}$ = 4.8 and 30 min at pH$_{in}$ = 6.8 at 25 °C. ATP synthesis was initiated by injection of 100 μl of this solution with a Hamilton syringe directly into the basic medium. Supplementary Table S2, supplement shows the resulting concentrations inside and outside the proteoliposomes after the acid–base transition during ATP synthesis. The increase of the ATP concentration was followed by the luminescence intensity. When the signal reached a constant level, it was calibrated by addition of an ATP standard solution. The internal pH was assumed to be equal to the pH measured after equilibration of 100 μl of the acidic medium with 15 μl of the proteoliposomes. The pH of the strongly buffered basic medium did not change after addition of the acidified liposomes, i.e. the pH$_{in}$ value was always 8.0. In addition to the transmembrane pH-difference a K$^+$/valinomycin diffusion potential was generated. The internal K$^+$ concentration was 0.6 mM, the external K$^+$ concentration was 106 mM. The transmembrane electric potential difference was estimated from the Nernst equation as 133 mV.

The luminescence time traces were fitted by a combination of an exponential and a linear function using the software package Origin. The initial rates were calculated from the fitted function. All given values are the arithmetic mean of triplicate measurements with the standard deviation.

3. Results

3.1. Subunit composition and oligomeric state of the isolated MF$_{0}$F$_{1}$

MF$_{0}$F$_{1}$ was isolated from yeast cells and purified as described in Materials and methods. Fig. 1A shows the results of the SDS-PAGE after the final purification step. The subunits were identified by mass spectrometric analysis and named according to the nomenclature of the Uniprot database. A comparison with earlier nomenclature is given in Supplementary Table S1. The oligomeric state of the isolated MF$_{0}$F$_{1}$ was analysed using blue native gel electrophoresis. Purified MF$_{0}$F$_{1}$ and for comparison MF$_{0}$F$_{1}$ solubilised from mitochondrial membranes with increasing ratios of Triton X-100 to protein were separated by BN-PAGE (Fig. 1B). At low Triton X-100 concentrations (lanes II and III) two dominant complexes were found, which were previously identified as monomeric and dimeric MF$_{0}$F$_{1}$, with the approximate molecular masses of 500 and 1000 kDa respectively [31,49]. At the highest Triton X-100 concentration (lane IV) the band attributed to the dimeric MF$_{0}$F$_{1}$ had disappeared, as reported previously [31]. Densitometric analysis of the gel revealed approx. 60% of the dimeric form in lane II. The DDM solubilised, purified enzyme (lane I) showed less than 1% of the dimeric form. We conclude that our purified MF$_{0}$F$_{1}$ only contains the monomeric form.

3.2. Subunit composition by HPLC–electrospray mass spectrometry

The SDS-PAGE gel of MF$_{0}$F$_{1}$ was analysed by HPLC–electrospray mass spectrometry as described in Materials and methods. Fig. 2A shows the analysed lane and its optical density profile. The distribution profiles for the MF$_{0}$F$_{1}$ subunits are given in Fig. 2 B–D, and show that the preparation contained all subunits except subunit K. Most of the subunits have been identified with sequence coverage above 60% as summarised in Table 1. Subunits 8, a, g and 9 have been detected with a lower sequence coverage which could be due to the hydrophobic nature of protein subunits 8, a and 9 and the lysine-rich sequence of subunit g. Subunit 9 was found in our SDS-PAGE at a molecular weight of 84 kDa (see Table 1 and Fig. 1), indicating that it was present as an oligomer as described earlier [31,50]. Surprisingly, and at variance with what has been found in previous preparations of yeast MF$_{0}$F$_{1}$ [12,51], the dimer-specific e- and g-subunits were also detected. Subunit g was identified with one peptide at the apparent molecular weight of 21.7 kDa (Table 1 and Fig. 2B, grey solid line). This is about twice as much as the calculated molecular weight of the mature protein, and also twice the apparent molecular weight reported previously [31,52]. Inspection of the HPLC–MS ion chromatograms showed that the mass of the identified peptide ion was also present at approximately 11 kDa, however at a lower intensity (Fig. 2, grey dashed line). Therefore, it appears that subunit g is present both as a dimer, as found in rat MF$_{0}$F$_{1}$ [53], and as a monomer.

Due to its high sensitivity, the mass spectrometric analysis allowed to detect also subunits of MF$_{0}$F$_{1}$ which were not visible in the Coomassie stained SDS-gel, either due to poor staining, or to co-migration in a single band (see e.g. subunit e, J and 8 in Fig. 2). In addition to the MF$_{0}$F$_{1}$
subunits, we also identified a number of peptides from other mitochondrial proteins, which were neither visible in the Coomassie nor in the silver stained SDS-gel (Fig. 1).

3.3. ATP synthesis by acid–base transitions

After reconstitution of MF0F1 in phosphatidylcholine/phosphatidic acid liposomes, acid–base transitions were carried out and ATP synthesis was measured as described in Materials and methods. Fig. 3 shows some original traces from these experiments. Fig. 3A was obtained at an internal pH of 5.0 and an external pH of 8.0 ($\Delta \phi = 133$ mV). The baseline resulted from the ATP content of the commercial ADP present in the basic medium. The acidified proteoliposomes were injected at time t=0 (indicated by the arrow). The injection resulted in a small mixing artefact, followed by an increase in luminescence due to ATP synthesis. The rate of luminescence increase was highest at t=0, decreasing to zero after approximately 15 s. This decrease of the rate is due to the decay of the transmembrane protonmotive force after the acid–base transition. The initial rate, i.e. the slope at $t=0$, was $v = 175$ nM s$^{-1}$ (see Fig. 3A). By taking into account the MF0F1 concentration in the reaction assay (2.0 nM), this gives a turnover value of $v/E_0 = 88$ s$^{-1}$.

The total amount of ATP generated in the acid–base transition (ATP yield) is also shown ($\Delta$ATP$_{total} = 175$ ATP per MF0F1). When the transmembrane $\Delta \mu H^+$ was abolished by addition of 50 mM NH$_4$Cl to the basic medium, no ATP synthesis could be detected (Fig. 3B). When $10$ $\mu$g/ml oligomycin, which blocks proton flow by binding to MF0F1, was added to the acidic and the basic medium, again no ATP synthesis could be observed (Fig. 3C).

3.4. Catalytically active MF0F1—monomer or dimer?

As shown in Fig. 1B, the purified MF0F1 was obtained in its monomeric form. During reconstitution of MF0F1 into preformed liposomes, detergent is added to destabilize the bilayer membrane and the hydrophobic parts of the MF0F1 micelles interact with the lipidome membrane. Removal of the detergent by BioBeads leads to an integration of the enzyme into the membrane with the liposome surface. The average number of MF0F1 per liposome can be estimated as follows. As determined by photon correlation spectroscopy [55], the average diameter of the proteoliposomes is 150 nm. Assuming that the thickness of the membrane is 8 nm (sum of the inner ($r_i$) and outer ($r_o$) surface, $O = 4\pi (r_i^2 + r_o^2) = 4\pi (67^2 + 75^2)$ nm$^2 = 1.3 \times 10^5$ mm$^2$), a surface area of 1.3 $10^5$ mm$^2$ is obtained. The average area of a lipid molecule is 0.6 nm$^2$ [56], i.e. an average liposome contains 2.2 $10^5$ lipid molecules. The lipid concentration during reconstitution was 8 mg/ml or 10.5 mM (calculated with an average molecular mass of the lipids of 760 g/mol) which corresponds to a liposome concentration of 48 nM.
The MF0F1 concentration during reconstitution was 150 nM. Assuming that all enzymes are reconstituted into the membrane, each liposome would contain on average 3 MF0F1. Based on these considerations, a dimerisation of MF0F1 in the membrane was possible under our conditions and, therefore, the rate shown in Fig. 3 might be due to both monomeric and dimeric enzymes.

To resolve this ambiguity, the MF0F1 concentration during reconstitution was varied from 0.1 to 10 MF0F1 per liposome, and the initial rate of ATP synthesis per MF0F1 was measured. While a significant dimerisation is unlikely in the range between 0.1 and 1 MF0F1 per liposome, it could occur in principle in the case of more than 1 MF0F1 are reconstituted per liposome. However, as shown in Fig. 4A, the turnover (rate per enzyme) did not depend on the number of MF0F1 per liposome in the whole range between 0.1 and 10 MF0F1 per liposome. In addition to the rate, the total amount of ATP per MF0F1 (ATP yield) generated in the acid–base transition (ΔATPtotal, see Fig. 3) was measured. It was constant in the range between 0.1 and 1 MF0F1 per liposome and decreased at higher ratios (Fig. 4B). Whereas the initial rate (turnover) depends only on pHout, pHin, Δφ and the substrate concentrations the ATP yield depends, additionally, on the

### Table 1

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*a ID from Uniprot database.

*b Molecular weight for gel slice as determined by extrapolation.

*c Calculated molecular weight of mature protein.

*d Sequence coverage of total sequence by peptides detected.

*e Maximum intensity (sum of peptide integrated ion currents).

*f Number of non-redundant peptides detected.

*g Q0B1V4 differs from swissprot id P05626 at positions Leu11 (Ala in P05626), which is within the transit sequence, and Ala171 (Arg in P05626) which has been identified (data not shown).

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**Fig. 3.** ATP synthesis catalyzed by MF0F1 liposomes. The ATP concentration was measured with luciferin/luciferase as a function of time after the acid–base transition. The acidified proteoliposomes were injected into the basic medium resulting in a small mixing artefact (gap). The arrows indicate the starting point of the reaction t=0. The slope directly after mixing (t=0) is the initial rate of ATP synthesis, calculated from the fitted curve (solid line). ΔATPtotal is the amount of ATP synthesized in the acid–base transition after 15 s (ATP yield). (A) ATP synthesis after an acid–base transition at pHin = 5.0, pHout = 8.0 and Δφ ≈ 133 mV. (B) same conditions as in A but with addition of 50 mM NH4Cl in the basic medium. (C) Same conditions as in A but in the presence of 10 μg/ml oligomycin in the acidic and the basic medium.

**Fig. 4.** ATP synthesis activity as a function of the number of MF0F1 per liposome. Experimental conditions as described in Fig. 3. The number of MF0F1 per liposome was varied by changing the MF0F1 concentration during reconstitution in a range between 5 and 480 nM at constant concentration of liposomes (48 nM). Each point is the average of three experiments with standard deviation. (A) Initial rate of ATP synthesis per MF0F1 (turnover). (B) Total amount of synthesized ATP per MF0F1 (ATP yield) measured 15 s after the acid–base transition (see Fig. 3).
internal buffer capacity of the liposomes, i.e. on the amount of protons stored inside. After the acid–base transition, the protons stored in the inner aqueous phase flow back to the external phase via a basal proton flux (through the membrane) and a phosphorylation coupled proton flux (through MFOF1). If more than one MFOF1 is present in the membrane, the phosphorylation coupled proton flux is distributed between two or more enzymes, which decreases the number of protons available for each enzyme, and, accordingly, the ATP yield (amount of ATP synthesized per MFOF1). Therefore, the observed reduction of the yield indicates that, at stoichiometric ratios higher than 1 MFOF1 per liposome in the reconstitution medium, more than one enzyme was reconstituted per liposome.

Comparing the ATP synthesis rate and the ATP yield as a function of MFOF1 concentration, we conclude that monomeric MFOF1 catalyzes high rates of proton transport driven ATP synthesis and that dimerisation, if it occurs, does not influence the ATP synthesis.

3.5. Optimization of reaction conditions for ATP synthesis

To obtain high rates of ATP synthesis, the reaction conditions of the acid–base transition have been optimized with respect to pHin and pHout. The electric potential difference \( \Delta \varphi = 133 \text{ mV} \) as well as the substrate concentrations were kept constant \([\text{Pi}] = 5 \text{mM}, [\text{ADP}] = 0.4 \text{mM}\). The incubation time of the proteoliposomes in the acidic medium was varied and the time chosen for further measurements was the time at which the rate showed no further increase with increasing incubation time. The incubation times were 30 min for 6.5 < pHin < 6.8, 10 min for 6.2 < pHin < 6.4, and 2 min for 4.8 < pHin < 6.0. The longer incubation time needed at the highest pHin was expected due to the lower concentration of the most permeant neutral and monoanionic forms of succinate. The dependence of the rate of ATP synthesis on pHin at pHout = 8.0 is shown in Fig. 5A. The rate shows a sigmoidal dependence on pHin reaching a maximal rate of 80 s\(^{-1}\) at pHin = 5.0. Incubation in the acidic medium might lead to an inactivation of the enzyme, especially at the highest proton concentrations. A small inactivation was observed only at the highest proton concentration (pHin = 4.8). This rate is shown by the open square in Fig. 5A. The inactivation has been corrected by comparing the rates at pHin = 5.2 with and without preincubation at pHin = 4.8, as described in Fig. S1 [57]. This correction increased the rate slightly (full square at pHin = 4.8). The observation of a maximal rate indicates that the enzyme has reached a fully protonated state, so that an increase of the proton concentration did not lead to a further increase of the rate.

The dependence of the rate of ATP synthesis on pHout at constant pHin = 5.2 is shown in Fig. 5B. With increasing pHout, the rate reached a maximum at pHout = 7.7 followed by a decrease. The increase in the rate with decreasing proton concentration outside indicates that at pHout = 7.2, the proton release from the enzyme to the outside is rate limiting and that this step is facilitated at low outside proton concentrations. However, the decrease of the rate at pHout > 8.0 was unexpected and cannot be explained by deprotonation of the enzyme. One possibility for this effect might be a limited supply of a substrate. In the measurements shown in Fig. 5A and B, the total phosphate concentration was kept constant, however, the relative concentration of the different phosphate species depended on the pHout and the observed decrease in the rate might be due to a limiting concentration of the phosphate species binding to the enzyme. This consideration prompted us to investigate the phosphate dependence of the rate in detail.

3.6. The phosphate species involved in ATP synthesis

The pHout determines the protonation state of substrates and products. It is still an open question which protonation state of phosphate binds to MFOF1 during ATP synthesis. Therefore, the rate of ATP synthesis was measured as a function of the phosphate concentration at different pHout. In Fig. 6A the relative rates of ATP synthesis are shown as a function of the total Pi concentration at different pHout between 7.2 and 8.6. At each pHout, the rate could be described by Michaelis–Menten kinetics:

\[
\frac{v}{v_{\text{max}}} = \frac{[\text{Pi}]_{\text{total}}}{K_M + [\text{Pi}]_{\text{total}}} + \frac{k_{\text{cat}} + k_1}{k_1}
\]

where \( K_M \) is the Michaelis–Menten constant referring to the total Pi concentration and \( v_{\text{max}} = k_{\text{cat}}[E_0] \) is the maximal rate. The solid lines in Fig. 6A were calculated from Eq. (1), and the parameters \( v_{\text{max}} \) and \( K_M \) were obtained from nonlinear regression analysis. The \( K_M \) values increased from 0.4 mM at pHout = 7.2 to 6 mM at pHout = 8.6 (see Fig. 6B). The maximal rates increased with pHout from 60 s\(^{-1}\) at pHout 7.0 to 120 s\(^{-1}\) at pHout 8.6 (see Fig. 7A). If the enzyme accepts only one protonation state of phosphate as substrate in ATP synthesis, the relevant parameter in kinetics is the concentration of this species, and not the total phosphate concentration. Therefore, the data was analysed as follows: Pi forms three ionic species in aqueous solution and the fraction of each species can be calculated from the dissociation constants of the three protonation states \( (K_1, K_2, K_3) \). The dissociation constants are corrected for the ionic strength of the reaction medium (\( I = 0.14 \text{ M} \)) as described in [58] resulting in \( pK_1 = 1.83, pK_2 = 6.89 \) and \( pK_3 = 12.07 \). For details see Supplementary Table S3.

Using these pK-values, the fraction of the monoanionic species \( H_2PO_4^- \) is calculated from Eq. (2) and shown as function of pHout in Fig. 6B.

\[
\alpha = \frac{[H_2PO_4^-]}{[\text{Pi}]_{\text{total}}} = \frac{K_1[H^+]^2 + K_1K_2[H^+] + K_1K_2K_3}{[H^+]^3 + K_1[H^+]^2 + K_1K_2[H^+] + K_1K_2K_3}
\]
For an appropriate description of the kinetics, the total \( P_i \) concentration in Eq. (1) was substituted by the \( H_2PO_4^- \) concentration.

\[
\frac{v}{v_{\text{max}}} = \frac{[P_i(\text{total})\alpha]}{K_{M\alpha} + [P_i(\text{total})\alpha]} = \frac{[H_2PO_4^-]}{K_M[H_2PO_4^-] + [H_2PO_4^-]}
\]

(3)

In this equation \( K_{M\alpha} \) is identical with the \( K_M \) for the species \( H_2PO_4^- \), i.e. \( K_{M\alpha} = K_M[H_2PO_4^-] \). The \( K_{M[H_2PO_4^-]} \) has been calculated from the data in Fig. 6B and plotted in Fig. 7B. \( K_{M[H_2PO_4^-]} \) did not depend on \( p_{H_{\text{out}}} \) and the \( K_M \) value for this species is \( K_{M[H_2PO_4^-]} = (120 \pm 20) \mu M \).

The \( H_2PO_4^- \) concentrations were then calculated from Eq. (2) for all data shown in Fig. 6A and such data were replotted in Fig. 8 as a function of the \( H_2PO_4^- \) concentration. The different dependencies on total phosphate concentration shown in Fig. 6A could now be described by a single function with \( K_{M[H_2PO_4^-]} = 120 \mu M \) (continuous line in Fig. 8). From this result we conclude that the substrate of MF0F1 in ATP synthesis is the monoanionic species \( H_2PO_4^- \).

The rate constant for \( H_2PO_4^- \) binding, \( k_1 \), can be estimated from the Michaelis–Menten kinetics under the conditions \( [S] \ll K_M \) and \( k_{\text{cat}} \gg k_{-1} \).

\[
v = \frac{v_{\text{max}}[S]}{K_{M} + [S]} = \frac{v_{\text{max}}}{K_{M}}[S] = k_1[S]
\]

(4)

A plot of \( \frac{v_{\text{max}}}{v} \) as a function of \( p_{H_{\text{out}}} \) is shown in Fig. 7C. A rate constant of \( 1.1 \times 10^8 \text{ M}^{-1} \text{ s}^{-1} \) is obtained at \( p_{H_{\text{out}}} > 8.0 \), below \( p_{H_{\text{out}}} = 8.0 \), the rate constant decreases, since the \( \Delta pH \) is too low to obtain the maximal rate. This indicates that the minimal rate constant for \( H_2PO_4^- \) binding is \( k_1 = 1.1 \times 10^6 \text{ M}^{-1} \text{ s}^{-1} \). Based on this value, the rate constant \( k_{-1} \) for \( P_i \) dissociation, and the dissociation constant \( K_D \), can be calculated from the definition of \( K_M \) and from the value of \( k_{\text{cat}} \left( k_{\text{cat}} = \frac{v_{\text{max}}}{K_{M[H_2PO_4^-]} = (120 \pm 20) \mu M \right) \), resulting in \( k_{-1} = 12 \text{ s}^{-1} \) and \( K_D = \frac{k_{-1}}{k_1} = 11 \mu M \), respectively.

4. Discussion

In this work, procedures are described to purify MF0F1 from yeast, to reconstitute it into liposomes and to measure high ATP synthesis activities. The turnover number (up to 120 s \(^{-1} \)) is in the same order of magnitude as that of bovine MF0F1 in SMP (50 s \(^{-1} \)) and of the
maximal turnover (440 s⁻¹), as estimated in [29], indicating that such isolation and reconstitution procedures largely preserve the native functional state of the enzyme. Since it is assumed that all MF0F1 added to the reconstitution mixture is inserted correctly into the liposome membrane and that all MF0F1 is active in ATP synthesis, this rate is the minimal turnover of our preparation. Although several isolation and (co-)reconstitution procedures have been reported, this is the first time a rate of ATP synthesis close to the estimated physiological value has been observed for the purified mitochondrial enzyme. Several reasons might be relevant for this high activity.

4.1. Subunit composition

The isolation procedure described here is similar to that reported in [12], and virtually the same band patterns were obtained by SDS-PAGE. Mass spectrometric analysis of the gel revealed that our preparation contained all subunits described previously [49,51–53,59,60] except for the K subunit [49]. Previous reports [12,31,51] had suggested that the subunits e and g dissociate from the complex when using high concentrations of DDM or Triton X-100 for solubilization of monomeric MF0F1. Notably, subunits e and g were also found in a preparation of bovine MF0F1 that was used for measurement of light-driven ATP synthesis [23]. These subunits have been shown to be involved in dimer/oligomer stabilization and to be not essential for oxidative phosphorylation in vivo [33,59,60]. Our data clearly show that subunit K is not necessary for high activities of the enzyme.

4.2. Monomeric and dimeric MF0F1

In recent years, evidence has been accumulating that MF0F1 is found in native membranes as dimers and even as oligomers (see [40] and references therein). The dimer-specific subunits (e, g and K) are found at the dimer interface and play a role in dimer stabilization and mitochondrial morphology. A still largely unexplored issue has been whether the monomeric and the multimeric forms are equally competent for catalysis or not. It may be possible that failure to detect high ATP synthesis rates in reconstituted systems was due to the fact that the monomeric enzymes were isolated. A higher efficiency of the multimeric forms in vivo has been suggested due to their ability to give rise to inner mitochondrial membrane invaginations, which might be able to sustain a more elevated local protonmotive force [36,37]. As shown by the BN-PAGE analysis ofFig. 1B, the purified enzyme contained only the monomeric form, but it was not possible to exclude that dimerization took place during the reconstitution procedure, in which the added detergent (Triton X-100) was slowly removed by BioBeads. If more than one enzyme is reconstituted into detergent micelles are added and, following their adsorption at the membrane, the insertion is unidirectional, with the large hydrophilic part directed to the outside. Finally, the proteoliposomes with the correctly inserted protein are stabilized by removal of the detergent either by adsorption to BioBeads or by dialysis. After optimisation of the parameters for reconstitution we adopted the procedure described in Materials and methods.

4.4. Reaction conditions for acid–base driven ATP synthesis

The proton motive force (ΔpH) necessary for ATP synthesis was generated by an acid–base transition and therefore, the initial reaction conditions are well known. In mitochondria, the electric component Δφ of the protonmotive force is larger than the chemical component ΔpH. To obtain high rates, Δφ was generated by a large K⁺ concentration difference ([K⁺]in = 0.6 mM, [K⁺]out = 110 mM) in the presence of valinomycin. The permeability coefficient of the K⁺/valinomycin complex is large compared to those of the other ions, so that the diffusion potential (ΔΔφ = 133 mV) calculated by the Nernst equation is close to the actual value. An internal K⁺ concentration lower than 0.6 mM would not lead to a higher Δφ since the initial influx of a few K⁺ ions compensating the electric membrane capacity would bring the actual internal K⁺ concentration to the range of few hundred of μM. Also higher external K⁺ concentrations would not increase the actual value of ΔΔφ, since at higher [K⁺]in/[K⁺]out ratios the membrane is not strictly semipermeable and compensating fluxes of other ions would decrease the diffusion potential.

At pHout = 8.0, the rate depended in a sigmoidal way on pHout with a maximal value of 85 s⁻¹ reached at pHis 5.0. This dependency reflects the protonation of the enzyme from the inside. Incubation at pH<5.0 might lead to an irreversible denaturation of a fraction of the enzyme and a method is described for correction of this denaturation (see Supplementary Fig. S1). When the constant total phosphate concentration was considered, the dependency of the rate on pHout showed a maximum at pHout = 7.8 (Fig. 5A). This indicates the superposition of two opposing effects on the rate. A detailed analysis of the pHdependency of the Michaelis Menten parameters for phosphate, which took into account the different protonation states of the substrate, was consistent with the hypothesis that the bell-shaped dependency of the rate on pHout observed in Fig. 5B resulted from the superposition of an increase due to the deprotonation of the enzyme at the outside and of a decrease of the substrate H₂PO₄⁻ concentration with increasing pHout. After correcting for such decrease, the maximal rate vmax reflected only the deprotonation of the enzyme to the outside, and increased correspondingly in a continuous manner, reaching a maximal rate at pHout 8.6 (Fig. 7A).

4.5. Phosphate as substrate in ATP synthesis

The rate of ATP synthesis as a function of the total phosphate concentration can be described by Michaelis–Menten kinetics and both Km as well as vmax depend on pHout. When the concentration of H₂PO₄⁻ is calculated at different pHvalues and the rates are plotted as function of H₂PO₄⁻ concentration, the phosphate dependencies obtained at different pHout can be described by a single Michaelis–Menten kinetics with KM(H₂PO₄⁻) = 120 μM and vmax = 120 s⁻¹ (see
Our data show that the phosphate species bound as substrate during protonation state cannot be seen in the crystal structure. However, K. Förster et al. / Biochimica et Biophysica Acta 1797 (2010) 1828

We show here that monoanionic H$_2$PO$_4^-$ for proton transport driven ATP synthesis might be reached at a

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