The thermodynamic H\textsuperscript{+}/ATP ratios of the H\textsuperscript{+}-ATPsynthases from chloroplasts and Escherichia coli

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The H\textsuperscript{+}/ATP ratio is an important parameter for the energy balance of all cells and for the coupling mechanism between proton transport and ATP synthesis. A straightforward interpretation of rotational catalysis predicts that the H\textsuperscript{+}/ATP coincides with the ratio of the c-subunits to \( \beta \)-subunits, implying that, for the chloroplast and Escherichia coli ATP synthases, numbers of 4.7 and 3.3 are expected. Here, the energetics described by the chemiosmotic theory was used to determine the H\textsuperscript{+}/ATP ratio for the two enzymes. The isolated complexes were reconstituted into liposomes, and parallel measurements were performed under identical conditions. The internal phase of the liposomes was equilibrated with the acidic medium during reconstitution, allowing to measure the internal pH with a glass electrode. An acid–base transition was carried out and the initial rates of ATP synthesis or ATP hydrolysis were measured with luciferin/luciferase as a function of ATP synthesis or ATP hydrolysis were measured with luciferase as a function of ATP concentration. The kinetics of the ATPase, numbers of 4.7 and 3.3 are expected.

Results

To determine the H\textsuperscript{+}/ATP ratio in the absence of other interfering reactions taking place in energy transducing membranes, we set up a minimal chemiosmotic system containing only the H\textsuperscript{+}-ATP synthase and a lipid membrane. Liposomes from phosphatidylcholine/phosphatidic acid were prepared, and either F\textsubscript{b}F\textsubscript{c} or EF\textsubscript{0}F\textsubscript{1} was reconstituted into these liposomes with a stoichiometric ratio of \( \approx 1 \) F\textsubscript{b}F\textsubscript{c} or 3 EF\textsubscript{0}F\textsubscript{1} per lipid. The H\textsuperscript{+}/ATP ratio of the E. coli enzyme are found in the literature, pointing to values between 2 and 3.6.

ATPase | FOF1 | proteoliposomes | chemiosmotic theory

Membrane-bound H\textsuperscript{+}-ATP synthases play a central role in the energy metabolism of all cells. They catalyze the synthesis of ATP from ADP and inorganic phosphate (P\textsubscript{i}) in bacteria, chloroplasts, and mitochondria (1–4). In these organelles, electron-transport generates a transmembrane electrochemical potential difference of protons, \( \Delta \mu \text{H}^+ \), and the H\textsuperscript{+}-ATP synthase couples the \( \Delta \mu \text{H}^+ \)-driven proton efflux with ATP synthesis (5, 6).

All H\textsuperscript{+}-ATP synthases have a similar structure: the hydrophilic F\textsubscript{1} part with subunits \( \alpha_3 \beta_3 \gamma_\delta \epsilon \) contains the nucleotide and P\textsubscript{i} binding sites, and the membrane integrated F\textsubscript{0} part, with subunits ab\textsubscript{2}c\textsubscript{10} in Escherichia coli (I, II, III\textsubscript{14}, and IV in chloroplasts), contains the proton binding sites. The kinetics of the enzyme was described by the binding change theory, in which the cooperativity of the three catalytic binding sites is accomplished by \( \gamma \)-subunit rotation (7). The high-resolution x-ray structure of F\textsubscript{0} corroborated this theory showing that the centrally located \( \gamma \)-subunit interacts differently with the three \( \beta \)-subunits and that the catalytic site on each \( \beta \)-subunit has a different conformation (8). Rotation of the \( \gamma \)-subunit was observed with different experimental techniques (9–11).

Current models of the coupling of proton translocation to ATP synthesis assume that proton flow through the F\textsubscript{0} part drives a rotation of the ring of c-subunits against the a- and b-stator subunits; the connection of the c-subunits to the \( \gamma \) and \( \epsilon \)-subunits leads to rotation of the latter within the \( \alpha_3 \beta_3 \) barrel, which brings about the conformational changes at the catalytic sites required for ATP synthesis. To accomplish rotation of the c-ring, protons are assumed to enter the F\textsubscript{0} part through an access channel on one side of the membrane, bind to the essential glutamate (or aspartate) on subunit c, and leave it through an asymmetrically placed exit channel on the other side (12–15). Cross-linking data (16–18), single molecule visualization (19–21), polarization data (22), and FRET measurements (23) indicate that the ring of c-subunits in F\textsubscript{0} rotates together with the \( \gamma \) and \( \epsilon \)-subunit in F\textsubscript{1}.

This mechanism appears to directly predict the H\textsuperscript{+}/ATP ratio: It should be equal to the number of c-subunits, divided by the number of \( \beta \)-subunits. Whereas all known F\textsubscript{0}F\textsubscript{c} ATP synthases contain three \( \beta \)-subunits, the number of c-subunits seems to be species-dependent. It has been reported to be 10 in mitochondria (24), 14 in chloroplasts (25), 10 in E. coli (26) and PS3 (27), 11 in the Na\textsuperscript{+}-ATP synthase from Ilyobacter tartaricus (28), and 13 in Bacillus sp. strain TA2A1 (29). The unexpected \( \beta \)-c stoichiometric ratio mismatch (pointing to a nonintegral value of the H\textsuperscript{+}/ATP ratio) and species specificity have stimulated anew much interest in this number (see, e.g., ref. 30).

The H\textsuperscript{+}/ATP ratio is important not only for understanding the coupling mechanism of the enzyme, but also for the energy balance of cells. Values of H\textsuperscript{+}/ATP between 2 and 4 have been reported and also variable ones (reviewed in ref. 31). The most recent data give H\textsuperscript{+}/ATP = 4 for the chloroplast enzyme (32–34), whereas H\textsuperscript{+}/ATP = 3 was reported for the mitochondrial enzyme (reviewed in ref. 35). Few data on the H\textsuperscript{+}/ATP ratio of the E. coli enzyme are found in the literature (31), pointing to values between 2 and 3.6.
\[
\text{ADP} + P_i \rightleftharpoons \text{ATP} + H_2O \quad [1]
\]
the Gibbs free energy of ATP synthesis is given by
\[
\Delta G_p = \Delta G_p^0 + RT \ln \left( \frac{[\text{ATP}]}{[\text{ADP}][P_i]} \right) = \Delta G_p^0 + 2.3RT \ln Q, \quad [2]
\]
where \(\Delta G_p^0\) is the Gibbs free energy in the biochemical standard state, \(Q\) is the stoichiometric product, and \(e^0\) is the standard concentration (equal to 1 M).

Proton transport from the internal to the external phase is described by the following equation:
\[
nH_{in} \rightleftharpoons nH_{out} \quad [3]
\]
with \(n = H^+/ATP\). Using the definitions \(\Delta pH = pH_{out} - pH_{in}\) and \(\Delta \varphi = \varphi_{in} - \varphi_{out}\), the transmembrane electrochemical potential difference of protons (Gibbs free energy of proton transport) is given by
\[
\Delta \mu_{H^+} = \mu_{H^+}(\text{in}) - \mu_{H^+}(\text{out}) = RT \ln [H_2^+] - RT \ln [H_\text{out}^+] + F \varphi_{in} - F \varphi_{out} = 2.3RT \Delta pH + F \Delta \varphi. \quad [4]
\]
The Gibbs free energy of the coupled reaction is the sum of Eqs. 2 and 4, i.e.,
\[
\Delta G' = \Delta G_p^0 - n \Delta \mu_{H^+} = \Delta G_p^0 + 2.3RT \ln Q - n2.3RT \Delta pH - F \Delta \varphi. \quad [5]
\]
At the point of equilibrium of the coupled reaction (\(\Delta G' = 0\)) and with \(\Delta \varphi = 0\), we obtain from Eq. 5:
\[
+ 2.3RT \ln Q = - \Delta G_p^0 + n2.3RT \Delta pH(\text{eq}). \quad [6]
\]
With experimentally determined \(Q\) and \(\Delta pH(\text{eq})\), the unknown parameters in Eq. 6 are the \(H^+/ATP\) ratio, \(n\), and the phosphorylation standard free energy, \(-\Delta G_p^0\).

The different parameters in Eqs. 5 and 6 were determined as follows.

- \(\Delta \varphi\). An acid-to-base transition gives rise to a transmembrane ionic imbalance, which automatically generates a diffusion potential, \(\Delta \varphi\). We have short-circuited it by using the same high \(K^+\) concentration (50 mM) on both sides of the membrane in the presence of 10 \(\mu\)M valinomycin. Under these conditions, the contribution of all other ions to the Goldmann diffusion potential was negligible, and \(\Delta \varphi = 0\).

- \(\Delta pH\). For reconstitution of the enzyme, the liposome membrane was permeabilized by addition of detergent. We assume that, during this process (2 h), a full equilibration of all ion concentrations between the internal and external phases has taken place, so that the composition of the internal phase is known. Correspondingly, the \(pH_{in}\) value was taken to be identical to the \(pH\) of the reconstitution buffer, which was measured with a glass electrode after the reconstitution. The \(pH_{out}\) value resulted from the mixing of the acidic reconstitution buffer containing the proteoliposomes with the basic medium and was measured with the same glass electrode. The error of the absolute \(pH\) was considered to result from the limited precision of the calibration buffers, with a standard deviation of \(\pm 0.02\) pH units. We have varied the \(pH_{in}\) values at constant \(pH_{out}\), i.e., the error associated with \(\Delta pH\) was \(\pm 0.03\) pH units.

- \(\Delta pH(\text{eq})\). For each given \(Q\) value, the rates of ATP synthesis and ATP hydrolysis were measured as a function of \(\Delta pH\), and the \(\Delta pH(\text{eq})\) was interpolated as the value at which the rate was zero.

- \(Q\). The stoichiometric product \(Q\) was established by setting the ADP, ATP, and \(P_i\) concentrations to the desired values. Because we measured initial rates of ATP synthesis and ATP hydrolysis (slope at \(t = 0\)), substrate and product concentrations remained at their preestablished values. Therefore, the error in \(\ln Q\) was very small and was neglected in our analysis.

To measure the initial rates of ATP synthesis and ATP hydrolysis, an ATP-monitoring luciferin/luciferase mixture was added to the basic medium in a luminometer cuvette. Additionally, the baseline of luminescence was recorded indicating the ATP concentration in the medium at the preestablished \(Q\) value. At reaction time \(t = 0\), the acidic buffer with proteoliposomes was injected with a syringe, and the luminescence was measured for \(\approx 3\) min (see SI Fig. 5). The composition of the external phase resulted from the mixing of the basic medium with the acidic reconstitution buffer containing the proteoliposomes (see SI Table 1). The CF0F1 and EF0F1 concentrations in the reaction medium were 7.6 nM and 23 nM, respectively.

Some of the rate measurements with CF0F1 are shown in Fig. 1 Left. An increase of luminescence indicates ATP synthesis; a decrease indicates ATP hydrolysis. The arrows indicate the time point of injection of the proteoliposomes into the basic medium, i.e., the start of the reaction. Each panel shows the data obtained at the same constant stoichiometric product. The \(Q\) values and the corresponding concentrations of ADP, ATP, and \(P_i\) are collected in SI Table 2. The different curves within each panel refer to different \(\Delta pH\) values, and the slope of each curve at \(t = 0\) gives the initial rate in units of ATP per enzyme per second. In each panel, the initial rate switches from ATP synthesis at the highest \(\Delta pH\) to ATP hydrolysis at the lowest \(\Delta pH\). No change in ATP concentration was observed when the \(\Delta pH\) was dissipated by 10 \(\mu\)M nigericin, indicating both the absence of adenylate kinase activity and the absence of ATP hydrolysis when the enzyme was not activated by a \(\Delta pH\).

Similar measurements were carried out with EF0F1, using the same method. For both enzymes, reconstitutions were carried out in parallel, using the same buffers, mixing ratios, \(pH\) electrode, etc. The only difference was that the EF0F1 proteoliposomes contained a 3-fold higher average number of ATP-synthase molecules per liposome. Despite this higher enzyme concentration, the signal-to-noise ratio in the EF0F1 traces was significantly lower. Lower absolute rates in EF0F1 were expected, because the maximal ATP synthesis turnover of EF0F1 was 80 s\(^{-1}\) compared with 280 s\(^{-1}\) for CF0F1. Moreover, in the present measurements, the \(\Delta \varphi\) was clamped to 0 and the EF0F1 turnover decreases much stronger with \(\Delta \varphi\) than that of CF0F1 (37).

Fig. 1 Right shows rate measurements with EF0F1 from \(Q = 12\) to \(Q = 152\). For each stoichiometric product, ATP synthesis is observed at the highest \(\Delta pH\) and ATP hydrolysis at the lowest. In all ATP synthesis traces, the initial rate decreases and switches to ATP hydrolysis after \(5\)–20 s. This reflects the decrease of the initial \(\Delta pH\) due to proton efflux. The data in the presence of 10 \(\mu\)M nigericin indicate the absence of ATP hydrolysis and adenylate kinase activities; at \(Q = 152\), a constant ATP concentration is initially observed, followed by a slow decrease of ATP, i.e., the enzyme is initially inactive and is activated by binding and hydrolysis of ATP.

For both enzymes, the initial rates and their errors were evaluated by monoeponential fitting of the first part of the traces (see details in SI Fig. 6) and were plotted versus \(\Delta pH\) (see Fig. 2). At the thermodynamic equilibrium, catalysis switches from ATP synthesis to ATP hydrolysis, and this point, \(\Delta pH(\text{eq})\), was determined by interpolation.
The activity of CF\textsubscript{0}F\textsubscript{1} is regulated by a disulfide bridge in the \(\gamma\)-subunit and by \(\Delta p\text{H}\) (39–42). When CF\textsubscript{0}F\textsubscript{1} is in its reduced state—achieved in this work by incubation with DTT—the rate of coupled ATP hydrolysis is controlled by two opposing effects. With decreasing \(p\text{H}\), the back pressure of protons is decreased; thus, the hydrolysis rate should increase, but the fraction of active enzyme also decreases, thus decreasing this rate. Both effects can be seen in Fig. 1 \textit{Left}. When the \(p\text{H}\) is collapsed by nigericin, i.e., the enzyme is not activated, no hydrolysis is detected. An increase of the hydrolysis rate with increasing reaction time (i.e., with decreasing \(p\text{H}\)) can also be observed (see, e.g., trace at \(Q = 58, \Delta p\text{H} = 1.96\)).

Only active enzymes catalyze proton transport coupled ATP synthesis/hydrolysis. Whereas the rates depend on the number of active enzymes, the position of the equilibrium described by Eq. 6 does not. The rates were measured in this work close to equilibrium, so that the activation should not influence the interpolated \(\Delta p\text{H(eq)}\). Nevertheless, we corrected for the effect of activation as described in ref. 34 by taking into account the sigmoidal \(p\text{H}\) dependency measured in thylakoid membranes (40) (SI Fig. 7 shows the corrected rates as a function of \(p\text{H}\)). The interpolated \(\Delta p\text{H(eq)}\) values were, within error limits, the same for the original and the corrected data (see SI Table 1). Regulatory phenomena dependent on \(\Delta p\text{H}\) have been shown in EF\textsubscript{0}F\textsubscript{1} as well (43), although a quantitative \(\Delta p\text{H}\) dependency is not available. The EF\textsubscript{0}F\textsubscript{1} traces of Fig. 1 \textit{Right} are consistent with a kinetic control exerted by \(p\text{H}\): similar to CF\textsubscript{0}F\textsubscript{1}, no hydrolysis is detected when the \(p\text{H}\) is collapsed by nigericin, and an increase of the hydrolysis rate with increasing reaction time (i.e., with decreasing \(\Delta p\text{H}\)) can be observed in several traces. The \(\Delta p\text{H(eq)}\) determined in Fig. 2 for both enzymes and the corresponding \(Q\) values were then plotted as \(2.3RT\text{ln}Q\) versus \(2.3RT\Delta p\text{H(eq)}\) in Fig. 3 \textit{Upper}. According to Eq. 6, such a plot should yield a straight line with slope \(n\) (the thermodynamic

\textbf{Fig. 1.} ATP synthesis and ATP hydrolysis after generation of a transmembrane \(\Delta p\text{H}\). The basic medium (900 \(\mu\text{L}\)) with the preestablished \(Q\) value and with luciferin/luciferase was placed into a cuvette in the luminometer, and the baseline was registered. The acid-base transitions were carried out by injection of 100 \(\mu\text{L}\) of proteoliposomes, incubated in the acidic reconstitution medium at different \(p\text{H}\). The final \(p\text{H(out)}\) was 8.47 \pm 0.02, and the \(\Delta p\text{H}\) values are given next to each trace. The addition gives rise to an immediate jump of the baseline. For clarity, the baseline was shifted back to the original luminescence level. The luminescence increase indicates ATP synthesis, the decrease indicates ATP hydrolysis. The different panels refer to different stoichiometric ratios \(Q\) as indicated. The luminescence was calibrated by addition of standard ATP. The solid lines show a biexponential fit of the complete time trace. The initial rates and their errors are given in units of \(10^{-3}\text{ s}^{-1}\); they were calculated from the monoexponential fit of the first part of the traces, as detailed in SI Fig. 6. (Left) CF\textsubscript{0}F\textsubscript{1} proteoliposomes. The final CF\textsubscript{0}F\textsubscript{1} concentration was 7.6 nM. (Right) EF\textsubscript{0}F\textsubscript{1} proteoliposomes. The final EF\textsubscript{0}F\textsubscript{1} concentration was 23 nM.

The activity of CF\textsubscript{0}F\textsubscript{1} is regulated by a disulfide bridge in the \(\gamma\)-subunit and by \(\Delta p\text{H}\) (39–42). When CF\textsubscript{0}F\textsubscript{1} is in its reduced state—achieved in this work by incubation with DTT—the rate of coupled ATP hydrolysis is controlled by two opposing effects. With decreasing \(\Delta p\text{H}\), the back pressure of protons is decreased; thus, the hydrolysis rate should increase, but the fraction of active enzyme also decreases, thus decreasing this rate. Both effects can be seen in Fig. 1 \textit{Left}. When the \(\Delta p\text{H}\) is collapsed by nigericin, i.e., the enzyme is not activated, no hydrolysis is detected. An increase of the hydrolysis rate with increasing reaction time, i.e., with decreasing \(\Delta p\text{H}\), can also be observed (see, e.g., trace at \(Q = 58, \Delta p\text{H} = 1.96\)). Only active enzymes catalyze proton transport coupled ATP synthesis/hydrolysis. Whereas the rates depend on the number of active enzymes, the position of the equilibrium described by Eq. 6 does not. The rates were measured in this work close to equilibrium, so that the activation should not influence the interpolated \(\Delta p\text{H(eq)}\). Nevertheless, we corrected for the effect of activation as described in ref. 34 by taking into account the sigmoidal \(\Delta p\text{H}\) dependency measured in thylakoid membranes (40) (SI Fig. 7 shows the corrected rates as a function of \(\Delta p\text{H}\)). The interpolated \(\Delta p\text{H(eq)}\) values were, within error limits, the same for the original and the corrected data (see SI Table 1). Regulatory phenomena dependent on \(\Delta p\text{H}\) have been shown in EF\textsubscript{0}F\textsubscript{1} as well (43), although a quantitative \(\Delta p\text{H}\) dependency is not available. The EF\textsubscript{0}F\textsubscript{1} traces of Fig. 1 \textit{Right} are consistent with a kinetic control exerted by \(\Delta p\text{H}\): similar to CF\textsubscript{0}F\textsubscript{1}, no hydrolysis is detected when the \(\Delta p\text{H}\) is collapsed by nigericin, and an increase of the hydrolysis rate with increasing reaction time (i.e., with decreasing \(\Delta p\text{H}\)) can be observed in several traces. The \(\Delta p\text{H(eq)}\) determined in Fig. 2 for both enzymes and the corresponding \(Q\) values were then plotted as \(2.3RT\text{ln}Q\) versus \(2.3RT\Delta p\text{H(eq)}\) in Fig. 3 \textit{Upper}. According to Eq. 6, such a plot should yield a straight line with slope \(n\) (the thermodynamic
H+/ATP ratio) and axis intercept $-\Delta G_0^{\text{H+}}$. The fitting by linear regression of the CF0F1 data (Fig. 3 Upper Left) gave values of $n = 4.0 \pm 0.2$ and $-\Delta G_0^{\text{H+}} = (38 \pm 3) \text{kJ mol}^{-1}$. The fitting by linear regression of the corresponding EF0F1 data (Fig. 3, Upper Right) gave values of $n$ and $-\Delta G_0^{\text{H+}}$, which were loaded with a much higher uncertainty, mainly due to the limited range of $Q$ values and $\Delta \text{pH(eq)}$ obtained for EF0F1. Therefore, the CF0F1 dataset was used as a reference, and the $-\Delta G_0^{\text{H+}}$ resulting from their fit was included in the EF0F1 dataset. The linear regression of these data yielded a value of $n = 4.0 \pm 0.3$, i.e., very similar to the value obtained for CF0F1. The dashed line has been drawn by imposing $n = 3.3$ (with $-\Delta G_0^{\text{H+}} = 38 \text{kJ mol}^{-1}$), and the dotted lines indicate that the expected $\Delta \text{pH(eq)}$ values in this case should have been $\pm 0.5$ units higher.

Fig. 2. Rate of ATP synthesis and hydrolysis as a function of the transmembrane $\Delta \text{pH}$. The initial rates and their errors obtained from Fig. 1, and additional measurements, were plotted as a function of $\Delta \text{pH}$. Some error bars are not visible, because they are smaller than the size of the symbols. Each curve represents catalysis rates measured at a constant stoichiometric product, $Q$. The data were fitted, using their errors as weight, by a function that was the product of a linear function (describing the rate-force relation near equilibrium) and of a sigmoidal function (describing the $\Delta \text{pH}$ dependence of activation). The fitting functions were used to interpolate the point at which the rate is zero [$\Delta \text{pH(eq)}$]. (Left) CF0F1 proteoliposomes. (Right) EF0F1 proteoliposomes.

Fig. 3. Determination of the thermodynamic H+/ATP ratio. $\Delta \text{pH(eq)}$ was plotted versus the stoichiometric product $Q$ according to Eq. 6 (data from Fig. 2). (Upper Left) CF0F1 data (from Fig. 2 Left). The error associated with $\Delta \text{pH(eq)}$ has been taken to coincide with the error estimated for $\Delta \text{pH}$ determination by the pH electrode ($\pm 0.03$). The data were fitted by a straight line, using their errors as weight. The slope of the linear regression gives the H+/ATP ratio. The y intercept gives the standard Gibbs free energy $-\Delta G_0^{\text{H+}}$ at $p_{\text{Hout}} = 8.47$. The arrow marked as R&S indicates the value of $-\Delta G_0^{\text{H+}}$ published in ref. 43. (Upper Right) EF0F1 data (from Fig. 2 Right). The $-\Delta G_0^{\text{H+}}$ value (diamond) is the best-fitting parameter out of the CF0F1 data (Left) and has been included in the linear regression of the EF0F1 data. The data were fitted by a straight line, using their errors as weight. The slope of the linear regression gives the H+/ATP ratio. To account for the error on the $y$ axis of the $-\Delta G_0^{\text{H+}}$ value, two additional linear fits were calculated, using its two extreme values of 35 and 41; the resulting H+/ATP values were 3.7 and 4.3 respectively, whose difference from 4.0, being higher than the error resulting from each linear regression, has been taken as the error in the determination of this parameter. (Lower) The CF0F1 (circles) and EF0F1 (triangles) data from Upper are plotted together for better comparison; the squares are CF0F1 data from ref. 34, in which the $\Delta \varphi$ component has been converted in $\Delta \text{pH}$ units (15 mV, corresponding to 0.26 $\Delta \text{pH}$ units). The straight line is the regression line of Upper Left.
In Fig. 3 Lower, the CF0F1 (circles) and EF0F1 (triangles) data are plotted together for better comparison. Also plotted are the previously obtained CF3F1 data from (34) (squares); the latter data had been measured in the presence of a ∆pH component of 15 mV.

It is interesting that the data presented in Fig. 2 can also be analyzed in a reciprocal manner by plotting the rates obtained at constant ∆pH versus RT ln [c/ATP] and determining RT ln[c/ATP] by interpolation (SI Fig. 8). When the interpolated RT ln[c/ATP] data were plotted versus 2.3RT ln[H+], a straight line was obtained with a slope of n = 4.1 ± 0.2 and ∆G°p = 39 ± 3 for CF3F1 and a slope of n = 4.0 ± 0.3 for EF0F1 (SI Fig. 9).

**Discussion**

The aim of this work was to determine the H+/ATP ratio from two H+-ATP synthases, which show differences in the c/β stoichiometric ratio, in particular EF0F1 and CF3F1, for which the c/β stoichiometric ratios have been reported as 3.3 and 4.7, respectively. The thermodynamic parameters of proton transport coupled ATP synthesis/hydrolysis were measured under identical conditions for CF3F1 and EF0F1 and the H+/ATP ratio for both enzymes was obtained from the change of the equilibrium ∆pH with ln[c/ATP].

These identical experimental conditions allowed us to, for evaluating the EF0F1 data, the ∆G°p value, which was obtained with higher precision from the CF3F1 data, thereby lowering the error limits in the H+/ATP determination for EF0F1. In fact, because of the lower rate of EF0F1, these measurements could not be extended to the wide range of Q values required to determine with high precision both ∆G°p and H+/ATP. The analysis of the CF3F1 data resulted in ∆G°p = 38 ± 3 kJ mol⁻¹ and H+/ATP = 4.0 ± 0.2. Using this ∆G°p value, the EF0F1 data gave H+/ATP = 4.0 ± 0.3. It is evident that, similarly to CF3F1, the measured thermodynamic H+/ATP ratio in EF0F1 is not consistent with the reported subunit stoichiometric ratio (26).

The H+/ATP ratio of 4 obtained for CF3F1 is in accordance with earlier results (32–34), and ∆G°p is in accordance with data in ref. 44. Values of 2–3.6 have been reported for H+/ATP in EF0F1; however, these measurements included use of indirect probes (45) and assumptions for estimating ∆ΔμH+ and ∆G°H+ in whole cells (46, 47).

The reason for the surprising difference found here between the thermodynamic H+/ATP and the c/β stoichiometric ratio is not known yet. The thermodynamic H+/ATP ratio reflects the minimal number of H+ necessary for ATP synthesis (or hydrolysis) of one ATP at equilibrium. At high rates of ATP synthesis far from equilibrium, it is conceivable that additional protons have to be translocated through the enzyme for each synthesized ATP, e.g., slip protons, leading to what can be called a “kinetic” H+/ATP ratio. It might be that the c/β stoichiometric ratio is optimized for such high rates, which would explain the mismatch between that ratio and the thermodynamic H+/ATP ratio. However, earlier proton flux measurements carried out far from equilibrium with thylakoid membranes also gave H+/ATP = 4 (48), lending no support to this speculation.

Alternatively, these data might indicate that the number of c-subunits in the holoenzyme CF3F1 is different from that found in the isolated ring of subunit III. As to the E. coli enzyme, it has been suggested that the number of c-subunits in this organism might change in response to the prevailing metabolic conditions (31), which would imply that the subunit number is not definitively settled in this organism either. The discrepancy presently found between the thermodynamic requirements (H+/ATP = 4 for both enzymes) and the subunit stoichiometric ratio (c/β = 3.3 for EF0F1 and c/β = 4.7 for CF3F1) raises interesting questions for future research.

**Materials and Methods**

CF3F1 from spinach (Spinacia oleracea) was isolated as described in ref. 34. CF3F1 was obtained in a buffer containing 1.25 M sucrose, 30 mM NaH2PO4/NaOH (pH 7.2), 2 mM MgCl2, 0.5 mM Na2EDTA, and 4 mM dodecyl maltoside, with a protein concentration of 4.2 mg/ml, and stored at −80°C. EF0F1 from pBVU13 E. coli strain DX8 (Δunc) was isolated as described in refs. 49 and 50. The EF0F1 complex was obtained in a buffer containing 0.9 M sucrose; 10 mM Mes; and 10 mM Tricine/NaOH (pH 7.0). 0.5 mM MgCl2, 5 mM thioglycerol, and 10 g/liter octyl glucoside, with a protein concentration of 2.2 mg/ml, and stored in liquid nitrogen.

Liposomes from phosphatidylcholine and phosphatidic acid were prepared by dialysis and stored at −80°C (37). CF3F1 or EF0F1 was reconstituted into these preformed liposomes with 0.8% Triton X-100 and Biobeads (stored in 50 mM KC1), using buffers at 12 different pH values. These acidic buffers contained 25 mM Mops/NaOH, 25 mM Mes/NaOH, 50 mM KC1, 4.4 mM MgCl2 (3.15 mM + 1.25 mM from the dialysis buffer), and 2.5 mM NaH2PO4 plus 5 mM Tricine, 0.1 M NaCl, and 0.1 M DTT from the dialysis buffer. Because of Triton X-100 treatment during reconstitution, all ion concentrations can be considered to have equilibrated between the bulk phase and the internal proteoliposome phase. The pH was measured with a glass electrode after reconstitution and is referred to as pHi. The final enzyme concentrations were 80 nM for CF3F1 and 240 nM for EF0F1.

The ATP concentration was measured with luciferin/luciferase (34). All suspensions and solutions were equilibrated at room temperature (23°C). Valinomycin was added to proteoliposomes to a final concentration of 10 μM. A luminometer cuvette was filled with 20 μl of luciferin/luciferase kit; 349 μl of a solution containing 100 mM KC1 and 9 mM MgCl2; 1 μl of a solution containing 1 M DTT and 50 mM KC1; 200 μl of a solution at pH 8.60 (containing 0.5 M Tricine, 5 mM NaH2PO4, 4.5 mM MgCl2, 50 mM KOH, and 330 mM NaOH); and 330 μl of a solution containing different concentrations of ADP and ATP (final volume 900 μl). The reaction was started by injection of 100 μl of proteoliposomes into the cuvette placed in the luminometer. The pH value measured after this mixing was the pH of the external phase during the ΔpH transition (pHout). The resulting ion concentrations of the internal and external phases and the nucleotide concentrations are summarized in SI Tables 2 and 3. The final ATP and ADP concentrations were 25 ± 800 nM and 1.7 ± 16 μM, and the final pHi concentration was 1.25 mM. The luminescence signal was calibrated by three additions of 10 μl of an ATP standard (10⁻⁵ M) to each cuvette. The luminescence data were sampled in 55-ms intervals. In the graphical representation of the data in Fig. 1, ten 55-ms time intervals have been averaged to reduce the noise. To calculate the initial rates and their errors, the initial phases of the signals at the original time resolution were fitted with either a monoeponential or a linear function (using the software package Origin). SI Fig. 6 shows the luminescence time traces at the original time resolution, the fitted functions, and the residuals for one Q value (Q = 152).

Measurements of the rate of ATP synthesis under standard conditions (pHi = 4.8, pHout = 8.5, [K+]in = 50 mM, [K+]out = 180 mM) were carried out as described in ref. 37. Concentrations of ATP and ADP were measured as described in ref. 34. The ATP content in ADP solutions was 0.16%.

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Fig. 4. Scheme of the chemiosmotic system. Liposomes were made from phosphatidylcholine and contained 5 mol % phosphatidic acid. Their mean diameter was 120 nm, containing $1.3 \times 10^5$ lipid molecules and one CF$_0$F$_1$ or three EF$_0$F$_1$. 

$pH_{\text{out}} = 8.50$
$[K^+]_{\text{out}} = 50 \text{ mM}$

$pH_{\text{in}} = 6.40$
$[K^+]_{\text{in}} = 50 \text{ mM}$
Fig. 5. Scheme of the experimental setup for measurements of ATP synthesis and ATP hydrolysis after generation of a transmembrane ΔpH by an acid-base transition. Proteoliposomes in the acidic reconstitution medium were injected into a cuvette in the luminometer containing the basic medium and the luciferin/luciferase reaction components, and the changes in luminescence were recorded after analog-to-digital conversion.
Fig. 6. ATP synthesis and ATP hydrolysis after generation of a transmembrane ΔpH at Q = 152, enlarged view. The luminescence time traces are shown here (after conversion to ATP/F0F1) at their original sampling frequency of 55 ms for CF0F1 and EF0F1 at Q = 152. For the purpose of error evaluation, the data were fitted with a monoexponential function using a nonlinear $\chi^2$ minimization routine (software package Origin). The $\chi^2$ was also evaluated as a function of time after the reaction start, and the appropriate time interval for the monoexponential fit was chosen as the time interval giving the minimal $\chi^2$. For the cases in which the monoexponential function degenerated to a straight line, linear regression analysis was applied instead. The resulting initial rates and errors are given for each trace. These initial rates and errors are depicted in Fig. 1 for all shown Q values, and they (and additional values) are shown in Fig. 2, with the corresponding error bars.
**Fig. 7.** Rate of ATP synthesis and ATP hydrolysis in CF$_0$F$_1$ as a function of the transmembrane $\Delta$pH after correction for enzyme activation. Each curve represents data at constant stoichiometric product Q. Data from Fig. 2 were corrected for activation as described in (34). After this correction, a linear relation between the rate and $\Delta$pH near equilibrium is observed, as expected from the thermodynamics of irreversible processes.
Fig. 8. Rate of ATP synthesis and hydrolysis as a function of $2.3RT\lg Q$. The initial rates obtained from Fig. 1 and additional measurements were plotted as a function of $2.3RT\lg Q$. Each line represents catalysis rates measured at a constant $\Delta pH$. Data were fitted by straight lines to determine the point at which the rate is zero ($2.3RT\lg Q(eq)$). (Left) $CF_0F_1$ proteoliposomes. (Right) $EF_0F_1$ proteoliposomes.
Fig. 9. Determination of the thermodynamic H⁺/ATP ratio from data analyzed at constant ΔpH. 2.3RTlgQ(eq) was plotted versus 2.3RTΔpH (Δμ_H⁺) according to Eq. 6 (data from SI Fig. 8). (Upper Left) CF₀F₁ data (from SI Fig. 8 Left). The slope of the linear regression gives the H⁺/ATP ratio. The y intercept gives the standard Gibbs free energy -ΔG^φ_f at pH_out = 8.47. (Upper Right) EF₀F₁ data (from SI Fig. 8 Right). The -ΔG^φ_f value (2.3RTlgQ at ΔpH = 0) is the best-fitting parameter out of the CF₀F₁ data (Left) and has been included in the linear regression of the EF₀F₁ data. The slope of the linear regression gives the H⁺/ATP ratio. Bottom: The CF₀F₁ (circles) and EF₀F₁ (triangles) data from Upper are plotted together for better comparison. The straight line is the regression line of the Upper Left.
Table 1. $\Delta p\text{H(eq)}$ values determined from Fig. 2 and corrected $\Delta p\text{H(eq)}$ values for CF$_0$F$_1$.

<table>
<thead>
<tr>
<th>$Q$</th>
<th>CF$_0$F$_1$ $\Delta p\text{H(eq)}$</th>
<th>CF$_0$F$_1$ $\Delta p\text{H(eq)}$ corrected</th>
<th>EF$_0$F$_1$ $\Delta p\text{H(eq)}$</th>
</tr>
</thead>
<tbody>
<tr>
<td>379</td>
<td>2.32 ± 0.03</td>
<td>2.34 ± 0.03</td>
<td></td>
</tr>
<tr>
<td>152</td>
<td>2.21 ± 0.03</td>
<td>2.21 ± 0.03</td>
<td>2.17 ± 0.03</td>
</tr>
<tr>
<td>58</td>
<td>2.11 ± 0.03</td>
<td>2.11 ± 0.03</td>
<td>2.12 ± 0.03</td>
</tr>
<tr>
<td>25</td>
<td>2.03 ± 0.03</td>
<td>2.05 ± 0.03</td>
<td>2.08 ± 0.03</td>
</tr>
<tr>
<td>12</td>
<td>1.90 ± 0.03</td>
<td>1.91 ± 0.03</td>
<td>1.94 ± 0.03</td>
</tr>
<tr>
<td>5.3</td>
<td>1.88 ± 0.03</td>
<td>1.89 ± 0.03</td>
<td></td>
</tr>
<tr>
<td>1.3</td>
<td>1.71 ± 0.03</td>
<td>1.70 ± 0.03</td>
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</table>

$\Delta p\text{H(eq)}$ data are from Fig. 2, $\Delta p\text{H(eq)}$ corrected data are from SI Fig. 7. The linear regression of the $\Delta p\text{H(eq) corrected}$ data resulted in $n = 4.0 \pm 0.2$ and $\Delta G^0_p = 38 \pm 2$ kJ·mol$^{-1}$. 

Table 2. Composition of the proteoliposomes suspension before (internal phase) and after (external phase) the acid-base transitions.

<table>
<thead>
<tr>
<th></th>
<th>Composition of internal phase</th>
<th>Composition of external phase</th>
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</thead>
<tbody>
<tr>
<td>MOPS (mM)</td>
<td>25</td>
<td>2.5</td>
</tr>
<tr>
<td>MES (mM)</td>
<td>25</td>
<td>2.5</td>
</tr>
<tr>
<td>NaOH (mM)</td>
<td>6 ÷ 30</td>
<td>67 ÷ 69</td>
</tr>
<tr>
<td>KCl (mM)</td>
<td>50</td>
<td>40</td>
</tr>
<tr>
<td>KOH (mM)</td>
<td>-</td>
<td>10</td>
</tr>
<tr>
<td>MgCl₂ (mM)</td>
<td>4.4</td>
<td>4.5</td>
</tr>
<tr>
<td>NaH₂PO₄ (mM)</td>
<td>2.5</td>
<td>1.25</td>
</tr>
<tr>
<td>Tricine (mM)</td>
<td>5</td>
<td>100.5</td>
</tr>
<tr>
<td>EDTA (mM)</td>
<td>0.1</td>
<td>0.01</td>
</tr>
<tr>
<td>DTT (mM)</td>
<td>- *</td>
<td>6</td>
</tr>
<tr>
<td>Valinomycin (µM)</td>
<td>100</td>
<td>10</td>
</tr>
<tr>
<td>pH values</td>
<td>5.84 ÷ 6.89</td>
<td>8.45 ÷ 8.49</td>
</tr>
</tbody>
</table>

* Dithiothreitol (50 mM) has been added to proteoliposomes 2 hrs prior to starting ATP synthesis/hydrolysis measurements.
Table 3. Stoichiometric product, Q for the reaction different conditions

<table>
<thead>
<tr>
<th>Q</th>
<th>ATP (nM)*</th>
<th>ADP (µM)</th>
<th>Pi (mM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>379</td>
<td>800 + 2.7</td>
<td>1.7</td>
<td>1.25</td>
</tr>
<tr>
<td>152</td>
<td>320 + 2.7</td>
<td>1.7</td>
<td>1.25</td>
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<tr>
<td>58</td>
<td>240 + 5.4</td>
<td>3.4</td>
<td>1.25</td>
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<tr>
<td>25</td>
<td>160 + 8.5</td>
<td>5.3</td>
<td>1.25</td>
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<tr>
<td>12</td>
<td>70 + 8.5</td>
<td>5.3</td>
<td>1.25</td>
</tr>
<tr>
<td>5.3</td>
<td>80 + 25.4</td>
<td>15.9</td>
<td>1.25</td>
</tr>
<tr>
<td>1.3</td>
<td>0 + 25.4</td>
<td>15.9</td>
<td>1.25</td>
</tr>
</tbody>
</table>

* Amount of ATP present in the ADP solution (0.16% ATP in ADP, as measured with the luciferin/luciferase assay.)