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# The rate of ATP synthesis by reconstituted $CF_0F_1$ liposomes

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**The conditions for reconstitution of $CF_0F_1$ into asolectin liposomes and for ATP synthesis driven by an artificially generated $\Delta\tilde{\mu}_{H^+}$ were optimized. A rate of maximally 200 ATP per $CF_0F_1$ per s was obtained with a multi-mixing quenched-flow technique. This rate is about half the maximal rate observed in natural thylakoid membranes.**

## Introduction

According to the chemiosmotic theory [1], a transmembrane electrochemical potential difference of protons, $\Delta\tilde{\mu}_{H^+}$, is sufficient to drive ATP synthesis by the membrane-bound ATPase, $CF_0F_1$, without involvement of other proteins. Thus, the rate of ATP synthesis after isolation and reconstitution of $CF_0F_1$ into liposomes should be as high as in the natural membrane. In order to obtain an energization, a system which generates an electrochemical potential difference of protons must also be incorporated into the vesicles. Bacteriorhodopsin [2,3] and Photosystem I [4] have been used for this purpose. However, only rates of less than 1% of the maximal in vivo rate were reported. It is not clear whether these low rates are due to experimental difficulties or to fundamental reasons, namely the fact that in vivo protons might migrate within the membrane to the coupling factor without involvement of the bulk phase. On the other hand, if a pH difference by an acid-base transition [5] is generated artificially, the $\Delta$pH is changing during the experiment and the yield of ATP is both dependent on the activity of the enzyme and the duration of the reaction (buffer capacity, $H^+$ permeability) [6]. In order to overcome these difficulties, we used a multimixing quenched flow technique which allows measurement of the ATP synthesis rate under conditions of constant $\Delta\tilde{\mu}_{H^+}$ [7].

## Materials and Methods

Chloroplast coupling factor complex $CF_0F_1$ was prepared as a protein-asolectin-Triton X-100 micelle, following the procedure of Pick and Racker [6], as described elsewhere [4].

Reconstitution of $CF_0F_1$ into liposomes were carried out by using a procedure similar to that described for the $H^+$-translocating ATPase of the thermophilic bacterium PS3 [8]. Soybean phospholipids (asolectin, from Associated Concentrates, U.S.A.) were partially purified before use [8] and stored at −80°C. 500 mg of this purified asolectin were suspended in 10 ml of solution (pH 8) containing 2% (w/v) sodium cholate/1% (w/v) sodium desoxycholate/10 mM Tricine-NaOH/0.1 mM EDTA/0.5 mM dithiothreitol. Cholic acid (Sigma) was recrystallized as

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Abbreviations: EDTA, ethylenediamino tetra acetate; $CF_0F_1$, proton-translocating ATPase from chloroplasts.

described earlier [9]. The asolectin-detergent solution was subjected to sonic oscillation in an ice bath for 4 × 30 s (Branson sonifier B12, at 20 kHz and 150 W) and either used immediately or stored at −80°C for up to 6 days.

The asolectin-detergent solution was mixed with $CF_0F_1$ micelles (1–7 mg/ml) so that the final protein concentration in the reconstituted vesicles was between 0.1–1 mg/ml and the final phospholipid concentration between 30–40 mg/ml. This mixture was dialyzed against 1 l of 10 mM Tricine-NaOH (pH 8)/2.5 mM $MgCl_2$/0.2 mM EDTA/0.25 mM dithiothreitol at 30°C for 12 h using a minilipoprep-dialyzer equipped with a Diachema membrane type 10.14 MWC 5000.

Protein concentration was determined after a modified Lowry method [10]. Addition of desoxycholate was found to be necessary for complete precipitation of the $CF_0F_1$ by trichloroacetic acid. $CF_1$-content of the total protein was measured by rocket immune electrophoresis [11,12]. The protein fractions used in our experiments contained between 85–100% $CF_0F_1$. The calculations were based on a mole mass of 410 kDa for $CF_1$ [13,14] and 112 kDa for $CF_0$ [15].

The acid-base transition for measuring the ATP yield under conditions where the $\Delta pH$ decays from its initial value to zero was carried out as follows: 10 µl proteoliposomes were added to 0.25 ml of the acidic solution 1, containing 30 mM succinate/5 mM $NaH_2PO_4$/2 mM $MgCl_2$/0.5 mM KCl/1 µM valinomycin, adjusted to the appropriate pH with NaOH. After 30 s incubation, 0.25 ml of the basic solution 2 (200 mM Tricine/120 mM KOH/5 mM $NaH_2PO_4$/2 mM $MgCl_2$/0.2 mM ADP, adjusted with NaOH to pH 8.7) was added giving a final pH of 8.4 after mixing with solution 1. After 15 s the reaction was stopped with 4% trichloroacetic acid and the ATP content was determined. For control, $CF_0F_1$ was added to a mixture of solution 1 and 2, incubated for 15 s and stopped as above. This value, which was mostly due to ATP contamination of ADP, was subtracted.

The rate of ATP synthesis was measured with a multimixing quenched flow apparatus (Durrum D 133) as described elsewhere [7]. The proteoliposomes were incubated in solution 1 (protein concentration, 5–50 µg/ml) in the syringe of the quenched flow apparatus. After 30 s incubation they were rapidly mixed in mixing chamber 1 with the basic solution 2. The reaction was allowed to proceed for a definite time (80 ms–10 s) and then stopped by addition of 4% trichloroacetic acid in the second mixing chamber.

The amount of ATP synthesized was measured either with the luciferin/luciferase technique or the $^{32}P$ method. In the experiments with $^{32}P$ radioactive phosphate (30 µCi/ml) was added to solution 2. The inorganic phosphate was precipitated with molybdate [16], and organic $^{32}P$ remaining in the supernatant was counted by Cerenkov radiation in a scintillation counter (Packard) as described earlier [17].

In the experiments with the luciferin/luciferase technique, 10 µl of the denatured reaction mixture was added directly to a test solution of 50 µl luciferin/luciferase assay (LKB kit) and 200 µl of a solution containing 0.1 M Tris-acetate (pH 7.75)/2 mM EDTA. The resulting luminescence was measured in an LKB luminometer 1250. The light output of each sample was calibrated by addition of an ATP standard. All reactions were carried out at room temperature.

## Results

For the optimization of the assay conditions the $CF_0F_1$ liposomes were incubated for 30 s in the acidic medium, mixed with basic medium, and after 15 s the reaction was terminated by addition of trichloroacetic acid. Fig. 1 shows the dependence of the ATP yield on the succinate concentration in the acidic medium. For the following experiments 30 mM succinate was used in each case. The reconstituted vesicles can be stored at 4°C for 8 days without decrease of the ATP yield. After about a fortnight, the ATP yield decreases to approximately half the original value. The reconstituted vesicles can also be frozen in liquid nitrogen, and after thawing they show an ATP yield of about 90% of that before freezing.

Fig. 2 shows the dependence of the ATP yield on the pH of the acidic medium; i.e., on $pH_{in}$. In the absence of diffusion potential the yield shows a maximum at about 4.9. If, in addition, a $K^+$/valinomycin diffusion potential, $\Delta\psi$, is generated, the yield is increased at all $pH_{in}$. For the

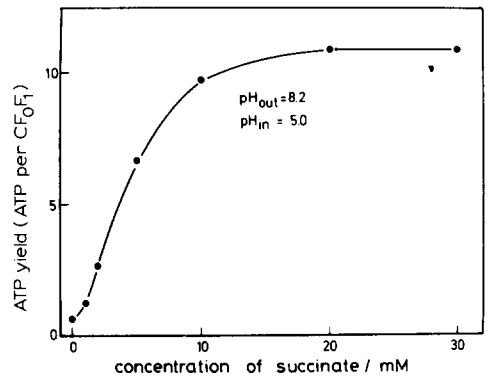


Fig. 1. Dependence of ATP yield on the concentration of succinate in the acidic medium. Reconstituted $CF_0F_1$ liposomes (10 $\mu$l with 1 mg/ml $CF_0F_1$) were incubated in 250 $\mu$l of acidic solution 1, containing the succinate concentration indicated. After 30 s 250 $\mu$l of basic solution 2 were added and the reaction was stopped after 15 s by addition of 0.5 ml of 4% (w/v) trichloroacetic acid. For further details, see Materials and Methods.

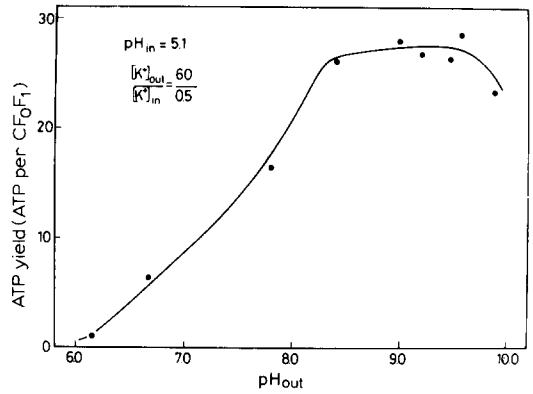


Fig. 3. Dependence of ATP yield on the $pH_{out}$. Experiments were carried out as described in Fig. 1, except that glycine was used instead of tricine and that $MgCl_2$ was added to the basic solution just before mixing.

following optimization we used a $pH_{in}$ around 5.0, supplemented with a diffusion potential.

Fig. 3 shows the dependence of the ATP yield on the pH of the basic medium; i.e., on $pH_{out}$. There is a continuous increase of the yield from $pH_{out}$ 6.0, reaching a plateau at about $pH_{out}$ 8.5. A decrease is observed above $pH_{out}$ 9.6.

Fig. 4 shows the ATP yield as a function of the incubation time in the acidic medium. While at pH 5.0 no change of the yield is observed between 10 and 60 s, the yield is decreasing rapidly at pH 4.4.

From these results the optimal conditions for testing the activity are $pH_{in}$ 5.0, $pH_{out}$ 8.5, additional diffusion potential, and incubation in the acidic medium between 10 and 40 s. These test conditions have been used in the following for optimization of the reconstitution procedure.

Fig. 5 shows the ATP yield when bovine serum albumin is added to the reconstitution medium.

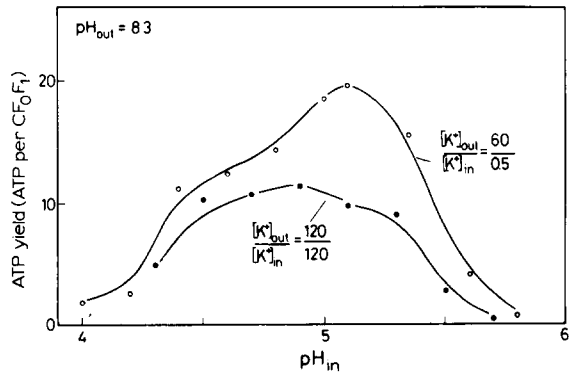


Fig. 2. Dependence of ATP yield on the pH of the acidic medium. Experiments were carried out as described in Fig. 1, except that the incubation time was 5 s and the succinate concentration was 30 mM. For curve A, 120 mM KCl were added to solution 1, giving no transmembrane difference of $K^+$ concentration. For curve B solution 1 contained 0.5 mM KCl, resulting in a ratio $[K^+]_{out}/[K^+]_{in} = 60/0.5$.

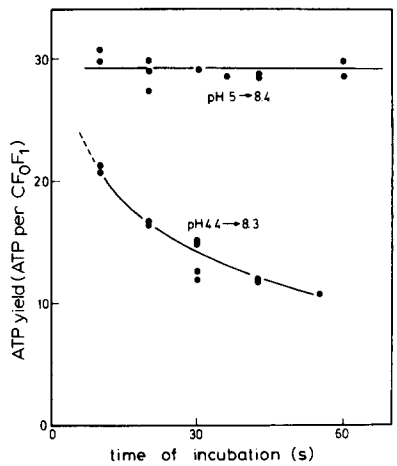


Fig. 4. Dependence of ATP yield on the incubation time in the acidic medium. Experimental details, see Fig. 1 and Materials and Methods.

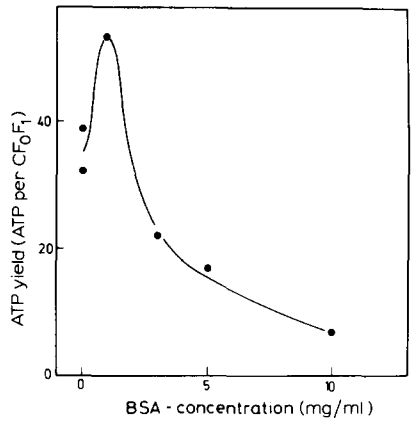


Fig. 5. Dependence of ATP yield on bovine serum albumin concentration during dialysis. Bovine serum albumin in the concentration indicated was added to the $CF_0F_1$ liposome-detergent mixture before dialysis. The ATP yield was measured under standard conditions; i.e., $pH_{in} = 5.0$, $pH_{out} = 8.4$, $[K^+]_{out}/[K^+]_{in} = 60/0.5$. For further details, see Fig. 1 and Materials and Methods.

The optimal bovine serum albumin concentration is 1 mg/ml.

Fig. 6 shows the time-course of the reconstitution during dialysis. After about 1.5 h, the half maximal yield is reached; between 3 and 4 h a plateau is reached where the yield does not change up to a dialysis time of 8–12 h. If the dialysis time is increased to 24 h the yield decreases again. This experiment has been carried out at 30°C.

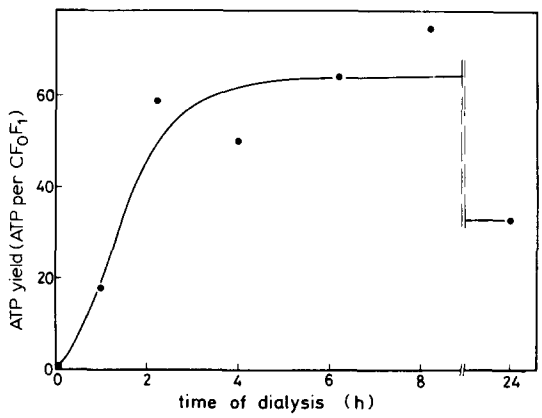


Fig. 6. Dependence of ATP yield on the dialysis time. The $CF_0F_1$ liposome detergent mixture, containing 1 mg/ml bovine serum albumin and 0.1 mg/ml $CF_0F_1$, were dialyzed at 30°C. ATP yield was measured under standard conditions (see Fig. 5).

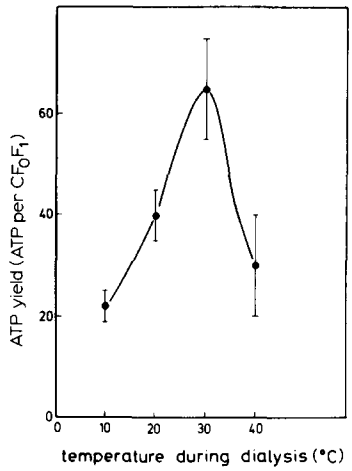


Fig. 7. Dependence of ATP yield on the temperature during dialysis. Data are from Fig. 6 and similar experiments at other temperatures. The ATP yield during the plateau phases is shown.

Fig. 7 shows the ATP yield reached in the plateau phase; i.e., between 4 and 12 h, as a function of the temperature during dialysis. Obviously, the optimal temperature for reconstitution is 30°C.

Table I shows a control experiment. The ATP yield was measured by the luciferase and by the $^{32}P$ technique. Both methods give the same results within error limits. Addition of triphenyltin chloride decreases the yield to 10% of the control.

TABLE I

ATP YIELD AND ITS INHIBITION BY TRIPHENYLTIN CHLORIDE MEASURED BY LUCIFERIN/LUCIFERASE AND $^{32}P$

TPT was added to the acidic medium 1. For the inhibition by EDTA the $CF_0F_1$ liposomes (0.1 mg/ml $CF_0F_1$ and 1 mg/ml bovine serum albumin were diluted 10-fold with water. After addition of EDTA (pH 8), the sample was incubated for 15 min and the ATP yield was determined. For further details see Fig. 1 and Materials and Methods.

| Conditions | ATP yield (ATP per $CF_0F_1$) | |
|---|---|---|
| | Luciferase | $^{32}P$ |
| Control | 26 ±2 | 31 ±7 |
| $+5 \cdot 10^{-5}$ M triphenyltinchloride | 2.8±1 | 2.4±3 |
| Control (+bovine serum albumin) | 49 | not determined |
| +2 mM EDTA | 15 | not determined |

EDTA treatment for 10 min after dilution of the proteoliposomes 1 : 10 with water reduces the yield to about 30%.

Fig. 8 shows the rate of ATP synthesis measured under the optimal assay and reconstitution conditions with the quenched-flow technique. On the left, the ATP yield is shown as a function of the reaction time. The slope of these curves in the linear range gives directly the rate of ATP synthesis. The lower curve was measured by the luciferase technique, the upper one with the $^{32}P$ technique. In both cases, a rate of approx. 100 ATP per $CF_0F_1$ per s resulted. On the right, a similar measurement with another batch of $CF_0F_1$ is shown. (Up to now, we have not systematically changed the isolation procedure for $CF_0F_1$). This is the highest rate which we have obtained until now with the reconstituted $CF_0F_1$ liposomes. The rates from the different batches were in a range from 80 to 200 ATP per $CF_0F_1$ per s.

If the amount of $CF_0F_1$ used in the reconstitution process is decreased by a factor of 10, the ATP yield is increased by 50%. The rate of ATP-synthesis is not changed significantly; however, the linear range of the curves extends to longer reaction times. From these results it may be concluded that the proton reservoir available for each ATPase is larger at low $CF_0F_1$ concentrations.

It should be mentioned that in one batch of $CF_0F_1$ the ATP yield in the acid-base transition

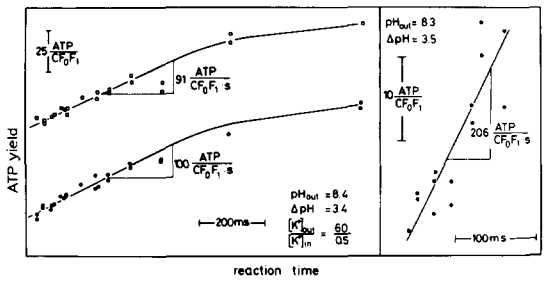


Fig. 8. ATP yield as a function of the reaction times. The slope of these curves give the rate of ATP synthesis, the numbers give the rate in ATP per $CF_0F_1$ per s. The curves are displaced arbitrarily from the origin. The first measurements were carried out 80 ms after mixing. Details see Material and Methods. Left ATP yield measured with luciferin/luciferase (lower curve) and with $^{32}P$ (upper curve). Right: ATP yield (luciferin/luciferase) with a different $CF_0F_1$ preparation.

under standard conditions using 0.1 mg/ml $CF_0F_1$ during the reconstitution was as high as 250 ATP/$CF_0F_1$. However, the measurement of the rate of ATP synthesis by the quenched-flow system revealed a rate of similar magnitude as described above. This rate was almost constant up to 1–2 s. This implies that not the activity of the $CF_0F_1$ was increased, but the basal permeability of the reconstituted liposomes was much smaller than usual. The reason for this effect is not yet clear.

## Discussion

This work shows that under optimal conditions the reconstituted $CF_0F_1$ liposomes give rates up to 200 ATP per $CF_0F_1$ per s. This is about half the maximal rate obtained by photophosphorylation [18,19] and by the quenched-flow technique with acid-base transitions in chloroplasts [7]. Any correction which should be applied (e.g., not all the $CF_0F_1$ added to the reconstitution medium is inserted into liposomes and, furthermore, not all ATPases which are inserted into the liposomes have the correct sidedness) would increase the rate per correctly incorporated $CF_0F_1$. Therefore, we came to the conclusion that the activity of the reconstituted $CF_0F_1$ is almost the same as in the natural thylakoid membrane.

Earlier experiments measuring the ATP yield by an acid-base transition [6] and with a diffusion potential [20] gave values of 32 [6] and 15 ATP per $CF_0F_1$ [20]. The optimization here increased the yield to about 60 ATP per $CF_0F_1$ per s. The rate of ATP synthesis in previous experiments was 0.4 [2], 0.5 [4], and 1.4 ATP per $CF_0F_1$ per s [3]. The high rate of ATP synthesis in our work has been obtained presumably because the following two difficulties of the earlier works have been avoided.

(1) In earlier works two membrane proteins, that is, the ATPase and a light-driven proton pump have to be incorporated into the liposomes. Therefore, the reconstitution procedure must be optimized for two different proteins, and this might be difficult.

(2) The energization of the membrane by the light-driven proton pumps is very low [3]. This might be due either to partial inactivation of the pumps or due to an incomplete orientation of the pumps in the membrane [4]. The low energization

then might be one of the reasons for the observed low rates of ATP synthesis.

The magnitude of the energization used for determination of the rate is not completely clear. Whereas the $\Delta pH$ is 3.4, the magnitude of the diffusion potential can be estimated only. If we assume that the liposome membrane is semipermeable for $H^+$ (in the presence of valinomycin), the diffusion potential is according to the Nernst equation 125 mV. A more realistic approach is the calculation of the diffusion potential according to the Goldmann–Hodgkin–Katz equation. However, the permeability coefficients for the different ions at this membrane are not known. If we use – just for an estimation – the permeability coefficients determined for $H^+$, $Na^+$, $Cl^-$ and $K^+$ (in the presence of valinomycin) at the thylakoid membrane, we obtain a diffusion potential of 60 mV. We assume that there is a diffusion potential in this range under standard conditions.

The data presented here do not answer the question, in which way protons are transferred from the electron-transport chain (source) to the ATPase (sink); i.e, via an intramembrane pathway [21] or via the internal bulk phase [1]. However, the data show that a bulk–bulk proton transfer is kinetically competent for the high rates of ATP synthesis observed in the natural thylakoid membrane. Furthermore, the high activity of reconstituted ATPase represents good starting material for further studies of the mechanism of ATP synthesis.

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