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Isolation and properties of the membrane-integrated part of the ATP-synthase from chloroplasts, CF_0*

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The ATP-synthase from chloroplasts, CF_0F_1, was isolated and purified and finally reconstituted into liposomes. The CF_1 part was solubilized from the CF_0F_1 proteoliposomes by treatment with NaBr. The resulting CF_0 proteoliposomes showed a proton conductivity of 16 H^+/CF_0 per s driven by a K^+/valinomycin diffusion potential. CF_0 can be removed from the liposomes by treatment with octyl glucoside. SDS-gel electrophoresis shows that CF_0 contains four different subunits, I, II, III, IV.

The membrane-bound H^+-ATPase from chloroplasts, CF_0F_1, catalyzes ATP-synthesis/hydrolysis coupled with a transmembrane proton flux. Like other ATP-synthases of the F_0F_1 type, it has a hydrophilic part, CF_1, containing the nucleotide-binding sites, and a hydrophobic part, CF_0, which is supposed to act as a proton channel. CF_1 contains five different subunits with the stoichiometry $\alpha_3\beta_3\gamma\delta\epsilon$ [1]. CF_0 contains four different subunits [2] with the minimal stoichiometry I, II, III_{12} IV [3]; however, this stoichiometry is still controversial.

To isolate CF_0, several different approaches can be used. Methods to deplete thylakoids from CF_1 by treatment with EDTA [4] or NaBr [5] have been known for a long time. However, the purification of CF_0 from thylakoid membranes seems to be difficult and the resulting preparations contain only three different subunits [6], i.e., subunit IV was lost during preparation.

Therefore, we decided to isolate and purify first CF_0F_1 from chloroplasts. The second step was reconstituted of the enzyme into liposomes, and then CF_0 and CF_1 were separated. This procedure leads to CF_0-proteoliposomes, which can be used directly in functional measurements. Finally, CF_0 was isolated from the liposomes by an octylglucoside treatment.

The ATP-synthase was isolated and reconstituted into asolectin liposomes as described earlier [3,7]. The resulting proteoliposomes were obtained in the dialysis buffer (10 mM Na-Tricine (pH 8.0), 2.5 mM $MgCl_2$, 0.25 mM dithiothreitol, 0.2 mM EDTA). They were centrifuged for 5 min at $13000 \times g$ (Beckman Microfuge) in order to remove aggregated lipids and large liposomes. The reconstituted protein is almost only found in the small liposomes. Protein content was measured according to Ref. 11.

Several chaotropic reagents (urea, guanidyl hydrochloride, NaBr) have been investigated for their ability to remove CF_1 from the proteoliposomes. Their concentration and the time of incubation have been altered. All these reagents were able to remove CF_1 at least partly from the liposome membrane. The following procedure proved to be the most efficient. The supernatant of the centrifugation described above is treated with 2 M NaBr at 0 °C for 30 min. CF_0 proteoliposomes are separated from the denatured subunits of CF_1 by centrifugation at $300000 \times g$ for 1 h. The pellet is washed wi' dialysis buffer, and the centrifugation is repeated.

For functional studies the pellet was resuspended in incubation buffer (1 mM Na-Tricine (pH 7.2), 2 mM $MgSO_4$, 200 mM choline chloride) and mixed with the same volume of potassium buffer (20 mM $NaHPO_4$, pH 7.2, 400 mM KCl). The proteoliposomes were loaded with potassium by sonication and freeze-thaw as described by Sone et al. [8]. The resulting suspension of

Abbreviations: CF_0F_1, protontranslocating ATPase ('ATP-synthase') from chloroplasts; CF_0, membrane-integrated part of ATP-synthase; octyl glucoside *n*-octyl β-D-glucopyranoside; SDS, sodium dodecylsulfate; FCCP, carbonyl cyanide-*p*-trifluorohydroxyphenylhydrazone.

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proteoliposomes now contains 0.5 mM N-Tricine, 10 mM $NaHPO_4$ (pH 7.2), 1 mM $MgSO_4$, 100 mM choline chloride, 200 mM KCl inside as well as outside and about 1 nM CF_0. 50 μl of this suspension was mixed with 3 ml incubation buffer, resulting in a final concentration of 3.3 mM K^+ outside.

This suspension was pipetted into a measuring chamber which was equipped with a glass electrode (type N, Schott) and a thalamide reference electrode (Schott). The pH was now recorded for 3 min, a time sufficient for stabilisation of the base line. Proton influx into the proteoliposomes driven by the K^+/valinomycin diffusion potential was initiated by addition of 1 μM valinomycin (final concentration) or 0.1 μM FCCP (final concentration). The resulting signal was filtered (Wavetek Rockland Model 452), amplified (Digistant type 6401), stored in a Nicolet Model 204 oscilloscope and finally displayed on a recorder (HP 7051). All measurements were carried out in a CO_2-free atmosphere in order to minimize a drift of the pH of the solution. The pH signals were calibrated by addition of 10 μl $3 \cdot 10^{-3}$ M HCl.

For isolation of CF_0 from the proteoliposomes the pellet of the second ultracentrifugation was resuspended in dialysis buffer. They were incubated for 45 min at room temperature with 25 mM octyl glucoside. After dilution with the 5-fold volume of dialysis buffer the sample is centrifuged for 1 h at 300 000 $\times$ g. The supernatant containing CF_0-micelles was lyophilized.

SDS-gelectrophoresis was run according to Laemmli [9] using 15% acrylamide in the separating gel and 3.75% in the stacking gel. The protein was dissolved in a sample buffer containing 125 mM Tris-HCl (pH 6.8), 40 g/l SDS, 10% glycerol (v/v), 10% mercaptoethanol (w/v). The samples were not heated before they were layered on top of the gel. After the run, gels were stained either with Coomassie Blue or silver [10].

Fig. 1 shows an SDS gel, stained with Coomassie blue, of the isolated CF_0 (lane 1) and CF_0F_1 (lane 2) and for comparison also CF_1 (lane 3). As is well known, CF_1 contains five subunits, designated α, β, γ, δ, ϵ. CF_0 contains four subunits, I, II, III and IV.

The SDS-gel electrophoresis was performed under mild conditions i.e., without heating the sample and in the absence of urea. Under these conditions subunit III runs on the gel as a 100 kDa band which presumably represents a dodecameric complex as has been demonstrated earlier with CF_0F_1 [3]. It can be seen in Fig. 1 that this complex is observed also with the isolated CF_0.

Subunit IV, which is homologous to subunit a from *E. coli*, is only weakly stained by Coomassie blue; but it is strongly stained with silver [2]. This may be the reason why the CF_0 subunit IV has not been detected in earlier attempts for isolation [6]. Only a few impurities can be seen on the gel. They seem to consist mainly of α, β and γ subunits of CF_1. Obviously not all CF_1 has

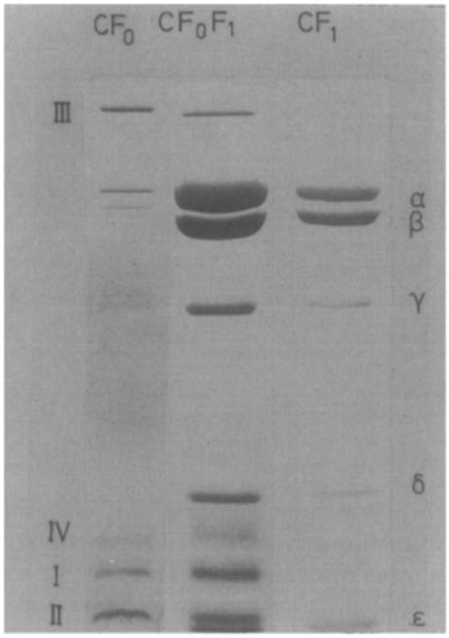

Fig. 1. SDS-gels of isolated CF (lane 1, 6 μg protein), CF_0F_1 (lane 2, 30 μg protein) and CF_1 (lane 3, 10 μg protein) after staining with Coomassie blue. Details, see text.

been extracted from the reconstituted CF_0F_1. Two minor bands can be detected between the β and γ subunits. These impurities are frequently detected in CF_0F_1 preparations, especially when sucrose density centrifugation is carried out with high protein concentration. A second treatment with NaBr similar to that described above does not improve the CF_1 depletion. Therefore, we assume that some of the CF_1 is oriented to the inside of liposomes and is not removed in the centrifugation step. From the stain intensity of the different bands it can be estimated that these impurities are less than 10% of total protein. Silver staining did not reveal any additional impurities.

The isolated CF_0 was investigated by electronmicroscopy after negative staining with 0.5% uranyl acetate. The electronmicrographs usually show one-dimensional ('strings') and two-dimensional aggregates of CF_0, lipids and detergent (octyl glucoside) and no internal structure can be detected in these aggregates. However, since presumably the hydrophobic parts of CF_0 tend to aggregate (as they do in CF_0F_1 [12]) the diameter of the strings corresponds to the membrane spanning length of CF_0. This length is 7.5 nm. It should be mentioned that the membrane-spanning length of the isolated subunit III complex is 6.1 nm [3]. This implies that the difference between the two dimensions results from the subunits I, II and IV. From the hydropathy patterns it results that subunit IV has five transmembrane helices with only very small hydrophilic stretches [13]. Subunits I and II have only one transmembrane helix each and both have large hydrophilic parts [13,14] and presuma-

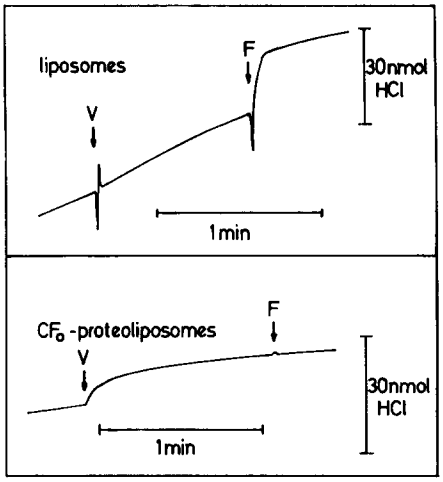

Fig. 2. pH of the liposome suspension in the reaction medium. V indicates addition of 1 μM valinomycin (final concentration); F indicates addition of 1 μM FCCP (final concentration). The upper trace shows a control experiment with liposomes. The lower trace shows an experiment with CF_0 proteoliposomes. Details, see text.

bly form the stalk between the membrane part and CF_1. However, a stalk-like structure cannot be seen on these strings.

CF_0 has different functions: it conducts protons across the membrane, it binds CF_1 and together with CF_1 it catalyzes proton transport coupled ATP-synthesis/hydrolysis. As a functional test for CF_0 we have measured proton conduction.

For these investigations, CF_0-proteoliposomes were loaded with KCl and the pH of the medium was continuously monitored by a glass electrode as described above. Fig. 2, bottom, shows the result of such a measurement: when valinomycin is added to these proteoliposomes a K^+ efflux is initiated and the proton concentration in the medium decreases. This indicates a proton influx into the proteoliposomes driven by a K^+/valinomycin diffusion potential. If additionally FCCP is added in order to increase the proton conductivity of the liposome membrane no further effect is observed. A control measurement is shown in the upper part of Fig. 2; in this case liposomes have been used which have been treated in exactly the same way as described for the proteoliposomes except that no CF_0F_1 was added during the reconstitution step. If valinomycin is added to this sample, no proton transport is observed. If now the proton permeability is increased by the addition of FCCP a significant proton influx is observed. These experiments show that the CF_0 proteolipsomes have a proton conductivity which is not observed in the liposomes without CF_0.

By calibration of the pH signal with HCl we can calculate from the initial slope, the rate of proton uptake. It results in 0.8 nmol H^+/s. Since the proteoliposomes in the reaction medium (3 ml) contain about 50 pmol CF_0 the result is 16 H^+/CF_0 per s. For this estimation it is assumed that about 90% of CF_1 could be removed from the proteoliposomes, and that all of the resulting CF_0 was active. If the latter assumption is not fulfilled, this will increase the rate per active CF_0. The observed rate has the same order of magnitude as found for other F_0 preparations (for review see Ref. 15). However, it is clear that for a proper function of CF_0F_1 the rate of proton translocation must be significantly higher (> 1000 H^+/CF_0F_1 per s), since the rate of proton transport coupled to ATP synthesis in the proteoliposomes is about 200 ATP/CF_0F_1 per s at $\Delta pH \approx 3.5$ [7]. Only for thylakoid membranes have sufficient high rates of proton transport been found up to now (10^5 H^+/CF_0F_1 per s [16]). We have to conclude, therefore, that the CF_0 is not in its native state in the liposome membrane. Obviously, the NaBr treatment does not only denature the CF_1 part, but also the CF_0 is at least partly denatured.

As a result, CF_0 in the liposome membrane is not very stable: when CF_0 proteoliposomes are stored in potassium buffer on ice for a couple of hours they lose their ability for valinomycin-induced proton uptake. This observation cannot be explained with the loss of proton permeability by CF_0, because addition of FCCP to the suspension does not result in a proton influx, either. This means that valinomycin can no longer produce any K^+ diffusion potential resulting in a proton influx. The only reason for this must be a dissipation of the difference between the K^+ concentration inside and outside the proteoliposomes during the 3 min that the pH electrode needs for equilibration after sample addition.

In order to show that the dissipation of the K^+ gradient across the proteoliposome membrane is not caused by a permeabilisation of the liposome membrane, CF_0F_1-proteoliposomes are loaded with KCl. When valinomycin and FCCP are added to a suspension of these CF_0F_1 proteoliposomes no difference occurs between the proton influx observed directly after preparation or after 24 h storage in potassium buffer.

The isolated CF_0 loses subunits I, II and IV completely after three freeze-thaw cycles (one freeze-thaw cycle is necessary for loading the CF_0-proteoliposomes with KCl), leaving behind the subunit III-complex. These observations together indicate that, after removal of CF_1, CF_0 becomes unstable. It dissociates into its subunits I, II and IV and the stable subunit III complex. Presumably, the subunit III complex has a K^+ conductivity.

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