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# Purification and characterization of the inhibitory subunit ($\delta$) of the ATP-synthase from *Micrococcus luteus*

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**Abstract** Subunit $\delta$ was isolated from the ATP-synthase from *Micrococcus luteus* strain (ATCC 4698). $\delta$, in the case of *M. luteus* $F_0F_1$-ATPase, acts as an inhibitor of ATP hydrolysis and thus resembles subunits in *E. coli* and chloroplast ATP-synthase. After treatment with 1.5 M LiCl the ATP-synthase dissociated, and subsequently subunit $\delta$ (27 kDa) was purified by hydrophobic interaction chromatography. Inhibition of ATP-synthase lacking $\delta$ by addition of $\delta$ showed non-competitve kinetics with a $K_i$ of ~5.9 nM. Subunit $\varepsilon$ from chloroplast $F_1$, which corresponds functionally to the *M. luteus* $F_0F_1$-$\delta$, and chloroplast $\delta$ were tested for ATPase inhibitory activity by addition to the partially $\delta$-depleted ATP-synthase from *M. luteus*. $CF_1$-$\varepsilon$ inhibited *M. luteus* ATP-synthase up to 80%, whereas $CF_1$-$\delta$ did not show any influence.

*Key words:* ATP-synthase; *Micrococcus luteus*; $CF_1$; $\delta$ subunit; $\varepsilon$ subunit; Inhibitor protein; Reconstitution

## 1. Introduction

ATP-synthases of mitochondria, eubacteria and chloroplasts synthesize ATP from ADP and $P_i$ by using a proton electrochemical gradient. The enzyme can also function reversibly to hydrolyze ATP and to generate a proton electrochemical gradient. $F_0F_1$-synthases consist of two parts, an intrinsic membrane component, $F_0$, conducting protons, and an extrinsic component, $F_1$, containing the catalytic sites for ATP-synthesis and hyrolysis [1,2].

The ATP-synthase is regulated by several inter-related factors, including the proton-motive force, the ATPase inhibitor protein and divalent cations [3]. $F_1$-ATPase inhibitor proteins have been isolated from bovine heart [4], *Saccharomyces cerevisiae* [5], rat liver [6], and plant mitochondria [7]. In the case of chloroplast and *Escherichia coli* ATP-synthases the $\varepsilon$ subunit of $F_1$ acts as an inhibitor of ATPase activity [8,9].

We have shown earlier that in *M. luteus*, subunit $\delta$ acts like an inhibitor of the $F_1$-ATPase and the $F_0F_1$ATP-synthase complex [10–12]. Apart from functional homologies the $\delta$ subunit from *M. luteus* shows N-terminal sequence homologies to the $\varepsilon$ subunits from other organisms [12].

In the present study, by using a new preparation of subunit $\delta$ and of ATP-synthase($-\delta$) [12], several aspects of the inhibitory subunit ($\delta$) were investigated. We also studied the interaction of the $\delta$ and $\varepsilon$ subunits from chloroplast $F_1$ with partially $\delta$-depleted ATP-synthase from *M. luteus*.

## 2. Materials and methods

### 2.1. Materials

LiCl was purchased from Fluka (Buchs), ATP from Serva (Heidelberg) and Tris (2-amino-2-[hydroxymethyl]-1,3-propandiol) from Boehringer (Mannheim). All other chemicals were at least of analytical grade and obtained from Merck, Darmstadt.

### 2.2. Isolation of the δ subunit from purified M. luteus ATP-synthase

*M. luteus* ATP-synthase was isolated as described previously [12]. The $\delta$ subunit was isolated from the purified ATP-synthase as follows. ATP-synthase (2 mg of protein) was dissociated by treatment with a chaotropic medium containing 1.5 M LiCl, 4 mM ATP, 50 mM Tris and 10% (v/v) glycerin at pH 7.5, for 30 min. The resulting mixture was applied to a hydrophobic interaction chromatography (HIC) column of Fractogel (R) EMD Propyl 650 (S). Material was eluted with a flow of 0.5 ml/min by a linear gradient of 100–0% LiCl. Protein was analyzed by the SDS-PAGE system of Laemmli [13] using a 12–15% linear acrylamide gradient.

### 2.3. Protein analysis

Protein was determined according to Lowry [14] and Dulley [15].

Dissociation products from the LiCl treatment of *M. luteus* ATP-synthase were analysed by Blue native PAGE of Schägger and von Jagow [16].

### 2.4. Purification of the small subunits from $CF_1$

Subunits $\delta$ and $\varepsilon$ from chloroplast $F_1$ were over-expressed in *E. coli* and purified as described [17].

### 2.5. Measurement of ATPase activity

ATP hydrolysis was measured via released phosphate from 1 mM Ca-ATP (5:1) according to the method of Fiske and Subbarow [18] with a continuous analysis system [19]. One ATPase unit hydrolyzed 1 $\mu$mol of ATP/min. The specific reaction conditions are given in the legends to the figures.

## 3. Results

### 3.1. Preparation of M. luteus $F_0F_1$–δ

Purified ATP-synthase from *Micrococcus luteus* was dissociated as described in section 2. Dissociation was controlled by native page. The mixture of dissociated $F_0F_1$ subunits was applied to a hydrophobic interaction chromatography-column of Fractogel (R) EMD 650 (S), and six peaks were eluted (Fig. 1). The first peak around 1.4 M LiCl contained subunits $\alpha$ and $\beta$, (Fig. 2), followed by undissociated $F_1$-ATPase at 1.2 M LiCl. $F_0F_1(-\beta)$ eluted in the third peak around 1.1 M LiCl, whereby the undissociated $F_0F_1$ was contained in peak four. Subunit $\delta$ eluted from the column at 0.7 M LiCl (peak 5); peak 6 contained a mixture of subunit $\gamma$ and $\beta$.

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*Abbreviations:* ATP-synthase (EC3.6.1.34) from *M. luteus*; ATP-synthase($-\delta$), ATP-synthase lacking the $\delta$-subunit; $F_0$, $F_0$ subcomplex of ATP-synthase; $F_1$, $F_1$ subcomplex of ATP-synthase; $CF_1$, chloroplast ATPase; $CF_0$, chloroplast proton channel; $CF_1$-$\varepsilon$, $\varepsilon$ subunit of chloroplast ATPase.


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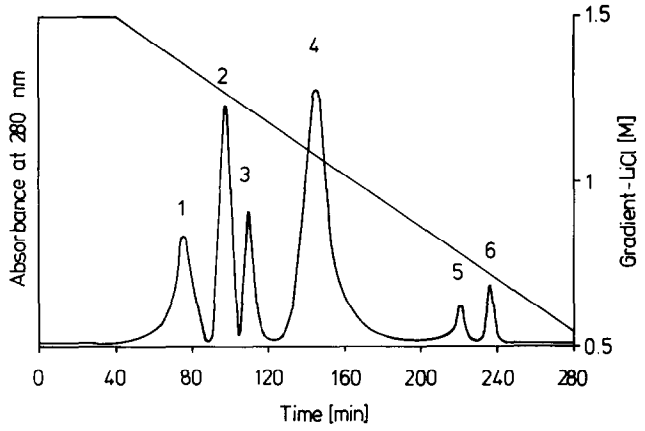


Fig. 1. Elution profile of hydrophobic interaction chromotography of 2 mg dissociated *M. luteus* ATP-synthase on Fractogel (R) EMD 650 (S). The sample was pretreated as described in section 2. Elution was by a linear gradient (1.5–0 M) of LiCl in 4 mM ATP, 50 mM Tris, 10% (v/v) glycerin, pH 7.5, at 0.5 ml/min. Absorption at 280 nm was detected and the salt gradient is shown as a straight line.

200 $\mu$g of subunit $\delta$ were obtained from 12 mg ATP-synthase. Since $\delta$ represents only about 5% of the total protein (540 kDa), the overall yield of the $\delta$ subunit is about 33%.

### 3.2. Reconstitution

Previously we have shown that the time-dependent increase in ATPase activity of *M. luteus* ATP-synthase is due to the slow dissociation of the $\delta$ subunit from the enzyme [12]. If the interaction between $\delta$ and the ATP-synthase($-\delta$) of *M. luteus* is

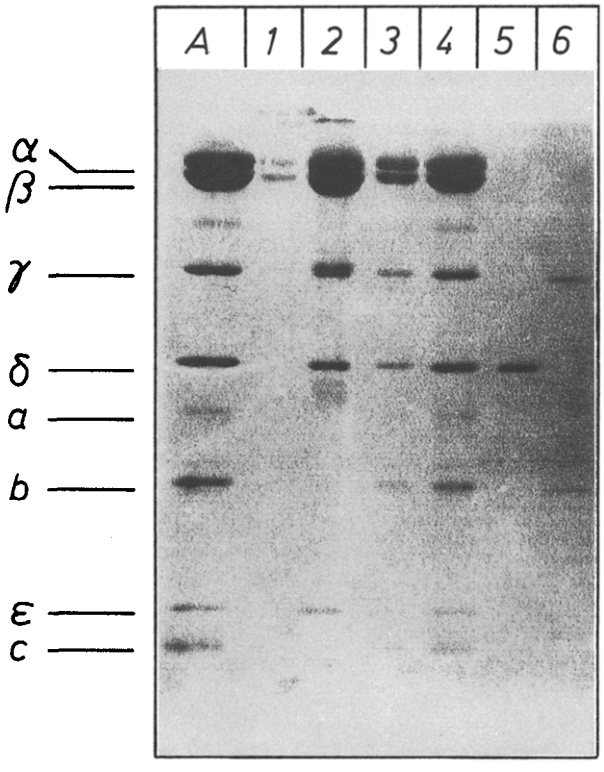


Fig. 2. SDS electrophoresis of samples obtained during preparation of dissociated *M. luteus* ATP-synthase. A 12–15% gradient gel (Coomassie blue stained) was run with the following samples: lane A, 20 $\mu$g of purfied ATP-synthase; lanes 1–6 correspond to peaks 1–6 in Fig. 1.

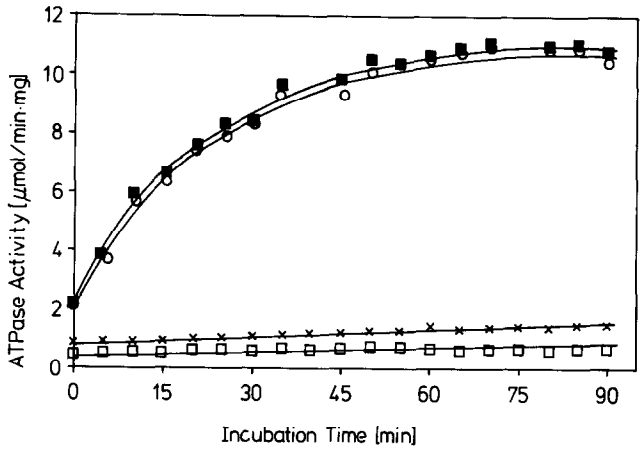


Fig. 3. ATP-hydrolysis after addition of $\delta$- (○) and $\varepsilon$ subunit (×) from $CF_1$ and $\delta$ subunit (□) from *M. luteus* ATP-synthase. The time-course of activation was measured by diluting 100 $\mu$g ATP-synthase with 100 ml of 0.1 M Tris-HCl (pH 8.0) at 37°C. Five minutes before the ATP hydrolysis was initiated by addition of 1 mM Ca-ATP, 1.0 $\mu$g of each subunit was added. The measurement without an addition of subunit is symbolized by (■).

governed by an association–dissociation equilibrium then inhibition should be reversed by addition of inhibitory subunit $\delta$. Fig. 3 shows that incubation of diluted ATP-synthase resulted in an increase in its hydrolytic activity, whereas addition of *M. luteus* $\delta$ subunit inhibited ATP-hydolysis by 90%. In analogy, hybrid reconstitution with $CF_1$-$\varepsilon$ reduced the rate of ATP-hydrolysis by about 80%. In contrast, no inhibition was observed by addition of the chloroplast $F_1$–$\delta$.

### 3.3. Kinetics of inhibition of ATP-hydrolysis by M. luteus δ

The effect of ATP concentration on ATPase activity was determined for ATP-synthase ($-\delta$) and *M. luteus* $F_0F_1(-\delta)$ plus a saturating concentration of $\delta$ subunit (Fig. 4). $V_{max}$ and $K_m$ values for uninhibited ATP-synthase($-\delta$) were 7.75 ∓ 0.45 units/ mg and 168.6 ∓ 6.7 $\mu$M, repectively. The values for $\delta$ subunit-inhibited ATP-synthase($-\delta$) were 2.99 ∓ 0.1units/ mg for $V_{max}$ and 159.7 ∓ 6.4 $\mu$M for $K_m$. These data demonstrate that the *M. luteus* $\delta$ subunit is a non-competitive inhibitor. A plot of the ATP-synthase($-\delta$) hydrolytic activity vs. $\delta$ subunit concentration (Fig. 4B) shows a maximal inhibition of 90%. Half-maximal inhibition occurs at a $\delta$-concentration of about 4.4 nM, $K_i$ = 5.9 nM.

## 4. Discussion

In the present work, the function of *M. luteus* has been studied by reconstitution experiments with purified $\delta$ and *M. luteus* ATP-synthase lacking $\delta$. Increase in the rate of ATP-hydrolysis by *M. luteus* ATP-synthase was reversed by addition of subunit and the chloroplast $CF_1$-$\varepsilon$, while addition of $CF_1$–$\delta$ was without effect.

The dissociation constant for the non-competitive inhibitor *M. luteus* $\delta$ with ATP-synthase($-\delta$) is in the nanomolar range, and enzyme in the presence of $\delta$ has about 10% of the activity of ATP-synthase lacking $\delta$.

Apparently, the $\delta$ subunit of *M. luteus* acts as an inhibitor of *M. luteus* ATPase just like chloroplast- and *E. coli* $F_1$-$\varepsilon$ [8,9,20]. In what way *M. luteus* $\delta$ occupies a role as a dissocia-

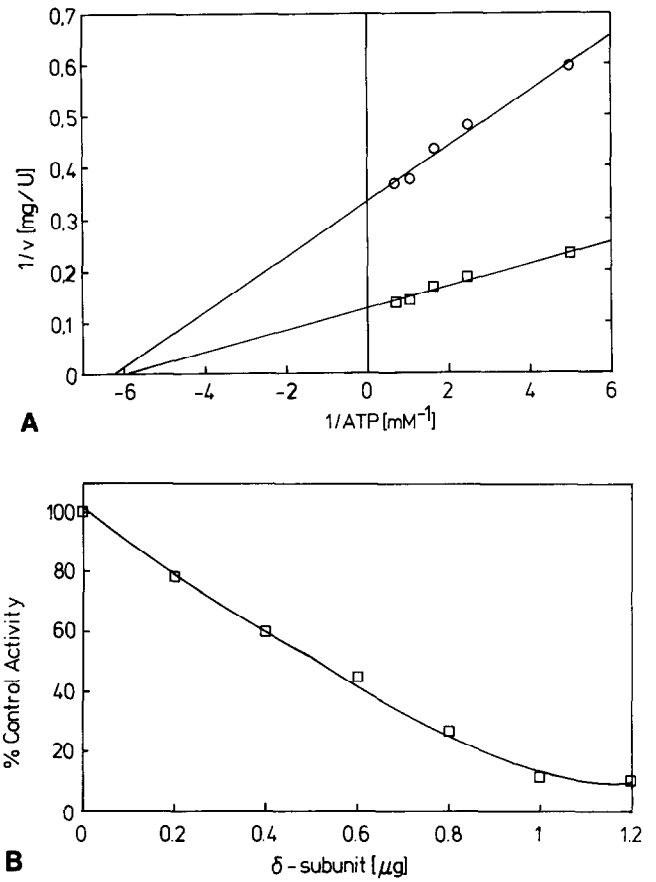


Fig. 4. (A) Effect of ATP concentration on ATPase activity of the inhibited and uninhibited ATP-synthase(−$\delta$). ATP-synthase(−$\delta$) was prepared as described [12]. 1 $\mu$g ATP-synthase was incubated in 0.1 M Tris-HCl (pH 8.0) at 37°C with (○) or without (□) 1 $\mu$g of $\delta$ subunit from *M. luteus*. After 5 min the ATPase reaction was started by addition of Ca-ATP and phosphate release was determined as described in section 2. (B) Activity vs. *M. luteus* $\delta$ concentration. 1 $\mu$g of ATP-synthase(−$\delta$) was incubated with varying concentrations of subunit $\delta$ from *M. luteus* and ATPase activities were determined as described in section 2. Control activity was 7.5 $\mu$mol·min$^{-1}$·mg$^{-1}$.

table inhibitor in the membrane-bound enzyme remains to be investigate. However, indications of a reversible dissociation/association of the $\delta$–ATP-synthase(−$\delta$) complex are revealed by investigations with reconstituted *M. luteus* ATP-synthase [12], where time-dependent incubation induced an increase in hydrolytic activity. In this context, it could be shown for the soluble ATP-synthase that the time dependence of ATPase activity is due to the dissociation of the $\delta$ subunit from the enzyme [12].

In comparison with *M. luteus* $\delta$, dissociation of chloroplast $\varepsilon$ from $CF_1$ is not a part of ATPase activation in the thylakoid membrane. The $\varepsilon$ subunit from chloroplast $F_1$ strongly interacts with the $\gamma$ subunit of $CF_1$ [21]. The electrochemical proton gradient induces changes in the position of $\varepsilon$ relative to the $\gamma$ subunit [21,22]. Furthermore, the $\varepsilon$ subunit from $CF_1$ is required for reconstitution of phosphorylation in the thylakoid membrane [23,24]. However, the fact that the ATPase activity of *M. luteus* ATP-synthase is inhibited by $\varepsilon$ subunit from $CF_1$ and $\delta$ subunit from *M. luteus* reveals their functional homology as an inhibitor.

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