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# Energy-dependent formation of free ATP in yeast submitochondrial particles, and its stimulation by oligomycin

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**Yeast submitochondrial particles, in a $P_i$- and NADH-dependent reaction, produced low concentrations of free ATP in the absence of added ADP. This formation of free ATP, as measured by the luciferin-luciferase method, was strongly stimulated by oligomycin. For maximal stimulation, oligomycin was to be added not earlier than 5–10 min after the addition of NADH. Upon addition of antimycin or FCCP the system was completely inhibited. The amount of free ATP formed corresponded to one-third of the amount of bound ATP in submitochondrial particles. The stimulatory effect of oligomycin disappeared if the submitochondrial particles were spun down after oligomycin stimulation and then resuspended in the reaction medium, whereas submitochondrial particles with no oligomycin added initially were stimulated by oligomycin after the same procedure. A different picture emerged with addition of ADP. If the submitochondrial particles were preenergized with NADH in the presence of oligomycin before the addition of ADP the formation of free ATP upon subsequent addition of ADP was inhibited by oligomycin. In the presence of oligomycin, but lacking preenergization with NADH, a stimulation of free ATP formation was achieved with added ADP. A possible explanation for the stimulating effect of oligomycin on ATP formation in the absence of added ADP is that it enhances the release of bound ATP in an energy-requiring process. The release of only about one-third of the bound ATP could indicate that one of three nucleotide-binding subunits involved in the mechanism of ATP formation by ATP synthase is in a state suitable for such an energy-dependent release of ATP.**

## Introduction

Bacterial, chloroplast and mitochondrial coupling factor ATPases (ATP synthases) are well known to have a protruding $F_1$ part, on which the phosphorylation and the ATP hydrolysis occur, and a more tightly membrane bound $F_0$ part, which constitutes the proton channel. The energy transfer inhibitor oligomycin has been a useful tool in a number of studies on the mitochondrial ATPase-complex. It binds to $F_0$, the membrane domain of the complex, and needs the oligomycin sensitivity conferring protein, OSCP, to inhibit the hydrolysis of ATP on isolated $F_1$ [1]. Oligomycin at low concentrations has been shown to act as a coupling agent in submitochondrial particles from bovine heart, apparently by tightening the leakage

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Abbreviations: $P_i$, orthophosphate; $PP_i$, pyrophosphate; FCCP, carbonyl cyanide *p*-trifluoromethoxyphenylhydrazan; DAPP, $P^1, P^5$-di(adenosine-5′)pentaphosphate; PMSF, phenylmethanesulfonyl fluoride.

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of protons through those $F_0$'s that are lacking $F_1$ [2], but when the concentration of oligomycin is raised, inhibition is obtained [3,4]. Recently it was demonstrated that DCCD and oligomycin when bound to $F_0$ reduce the affinity of the catalytic site of $F_1$ for ATP [5].

According to an alternating site mechanism for synthesis of ATP on the ATPase, in the three identical $\beta$-subunits of $F_1$, the binding of substrate to one $\beta$-subunit causes release of product at another, while the phosphorylation reaction is taking place at the third [6]. The formation of ATP from ADP and $P_i$ occurs as a single in line reaction without any phosphoenzyme intermediate, as has been shown in studies on the reverse reaction [7]. Energy may be required less for the ATP synthesis than for the release of ATP from the enzyme [8,9]. It was recently shown that energy input on bovine heart submitochondrial particles causes release of tightly bound ATP [10]. It is possible to synthesize enzyme bound ATP on soluble ATPase [11], and it appears that models of ATP synthesis, based on studies with soluble enzyme, hold also for $F_1$ bound to $F_0$ in SMP [12].

This paper deals with our investigation of the formation of free ATP from endogenously bound as well as added adenine nucleotides in yeast submitochondrial particles. We show that oligomycin in the absence of added ADP strongly stimulates formation of free ATP in an energy-(NADH) and phosphate-dependent reaction.

## Materials and Methods

Mitochondria were prepared from yeast (*Saccharomyces cerevisiae* strain AB310, NCYC1075), grown as in Ref. 13, by the enzymatic zymolyase method described therein, with the modifications that no PMSF was used and that 0.1% galactose and 2-mercaptoethanol were used in the buffer for the enzymatic digestion. Mitochondria were suspended in 0.35 M NaCl and 0.2 M glycylglycine (pH 7.4).

Submitochondrial particles were prepared from fresh mitochondria by sonication of a cold 20 mg protein/ml suspension (same buffer as before), with a Branson sonifier for $4 \times 30$ s, pulsed mode and 50% duty cycle. Mitochondria were removed by centrifuging the suspension for 15 min at $19000 \times g$. The supernatant was centrifuged for 40 min at $134000 \times g$ and submitochondrial particles were collected from the pellet, washed once and stored in liquid nitrogen. Protein was determined according to Ref. 14.

Continuous formation of free ATP was measured luminometrically according to Ref. 15. The assay buffer (buffer A) was 0.1 M Tris, 2 mM EDTA (pH 7.75) (acetic acid), 3 mM phosphate ($P_i$), 10 nM DAPP (to inhibit adenylate kinase activity) and ATP monitoring reagent, which contains $Mg^{2+}$ (10 mM final concn.), $PP_i$, bovine serum albumin, luciferin and luciferase (see Ref. 15).

For nucleotide determination submitochondrial particles were diluted in buffer A and precipitated with 0.5 M $(NH_4)_2SO_4$ (1 : 1) for 30 min, centrifuged at $12000 \times g$ for 15 min. The pellet was washed four times. Nucleotides were extracted from submitochondrial particles with 1 vol. 10% perchloric acid and 4 mM EDTA (0–4°C) to 1 vol. of resuspended pellet and incubated for 30 min (on ice). The suspension was centrifuged as above and the supernatant retained and neutralized with 2.5 M KOH. The precipitate was spun down and the supernatant was used for nucleotide determinations. AMP and ADP were converted to ATP using adenylate kinase, phospho*enol*pyruvate and pyruvate kinase. ATP was determined using the luminometric method [15].

## Results

### *The stimulating effect of oligomycin on formation of free ATP*

Yeast submitochondrial particles show, as determined with the luminometric method [15], without addition of ADP, a formation of low amounts of free ATP, which is dependent on $P_i$ and NADH as a suitable donor of reducing equivalents. Addition of oligomycin immediately stimulates the rate of free ATP formation (Fig. 1), sometimes more than 100-fold. This oligomycin-stimulated formation of free ATP can be seen even in preparations where no reaction occurs in its absence. The amount of free ATP formed (due to oligomycin) is in the concentration range 0.2–0.5 nmol/mg submitochondrial particles protein. The oligomycin concentration required for maximal

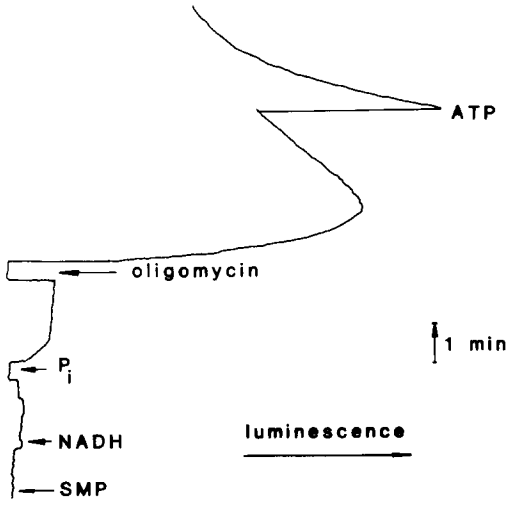


Fig. 1. A typical curve from the luminometric measurements of free ATP formed. The assay mixture contained (final concentrations): 3 mM phosphate, 5 mM NADH, 10 mM DAPP and ATP monitoring reagent (see Materials and Methods) in 0.1 M Tris-acetate, 2 mM EDTA (pH 7.75). The total volume was 1.0 ml. Additions: 160 μg submitochondrial particle protein, 20 μg oligomycin, 10 nM ATP standard.

stimulation is about 10 μg oligomycin/mg protein (Fig. 2). The NADH concentration required is around 2–5 mM. Preincubation of submitochondrial particles with oligomycin for 40 min at 23°C does not abolish this stimulation, neither does preincubation of submitochondrial particles with oligomycin and NADH before the start of the reaction with $P_i$.

Stimulation of ATP formation by oligomycin is only achieved once. Two samples of submitochondrial particles were pretreated with $P_i$, NADH and, in one sample, oligomycin, and pelleted. The supernatant was discarded and the ATP formation experiment was repeated, now with resuspended pellets. Only the sample lacking oligomycin during pretreatment showed stimulation of free ATP formation when oligomycin was added.

The low rate of formation of free ATP in the absence of oligomycin diminishes rather fast (about 90% in about 10 h) when submitochondrial particles are kept at 0 and −177°C, but the stimulation by oligomycin is retained. Thus, a 'selective ageing' is occurring. Oligomycin had a similar stimulating effect on the formation of free ATP also in other strains tested, such as the wild strains D273-10B/$A^{\alpha}$ ($met^-$) and PS 194 and the mutants PS 195, PS 211 and M28-81$^{\alpha}$ ($met^-$), which all have mutations in the OLI 1 or OLI 2 gene (giving lower, but not lacking, oligomycin sensitivity [16–18]).

Antimycin (10 μM), as well as FCCP (10 μM), inhibits oligomycin-induced formation of free ATP. Both antimycin and FCCP cause, upon addition, immediate hydrolysis of ATP. Controls were made to check for possible effects on the

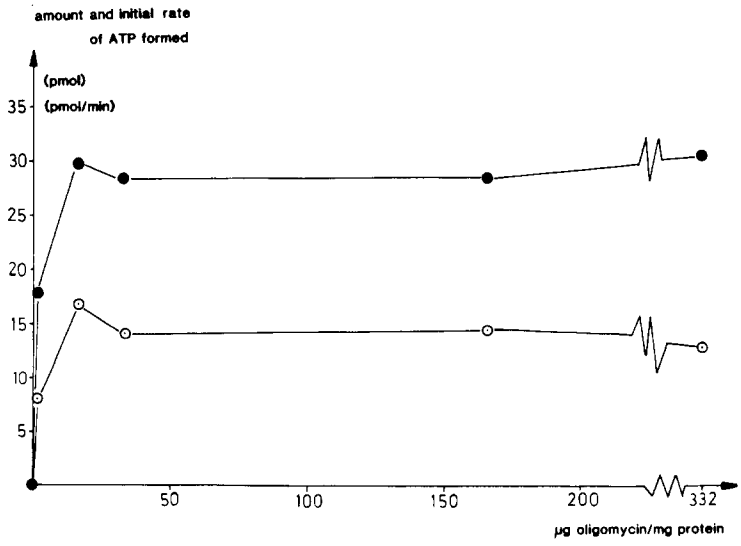


Fig. 2. Amount (filled circles) and initial rate (empty circles) of oligomycin-stimulated ATP formation of yeast submitochondrial particles as a function of oligomycin concentration as measured with the luminometric method (see Materials and Methods). Additions: 121 μg submitochondrial particle protein, 3 mM $P_i$ and 5 mM NADH. Total volume: 1 ml.

luciferase system of oligomycin, antimycin and FCCP. The two former gave no effect, whereas FCCP caused a 20% decrease in the sensitivity.

*Energy dependence of stimulation*

Stimulation by oligomycin addition is increased with the time after NADH addition before oligomycin is added. Optimal stimulation is achieved when oligomycin is added after about 5–10 minutes of energization with NADH (Fig. 3). The time needed for energization with 5 mM NADH does not seem to be dependent on the amount of submitochondrial particles. The amount of free ATP formed when oligomycin is added after 10 min of energization is plotted against different concentrations of submitochondrial particles (Fig. 4). It can be seen that less than 30 μg/ml submitochondrial particle protein gives a concentration of ATP that is proportional to the concentration of submitochondrial particles, with a proportionality constant of about 0.34 nmol ATP/mg protein. For higher concentrations of submitochondrial particles, a limitation in free ATP formation can be seen.

*Hydrolysis of ATP*

After the oligomycin-stimulated free ATP formation has reached its maximal level, hydrolysis of this ATP is observed (see Fig. 1). The rate of hydrolysis is also stimulated by oligomycin. As expected, addition of ATP increases the rate of hydrolysis (Fig. 1) and addition of ADP decreases it. The hydrolysis of added ATP is not stimulated by oligomycin. In fact, preincubation of SMP with oligomycin nearly completely inhibits the hydrolysis of added ATP.

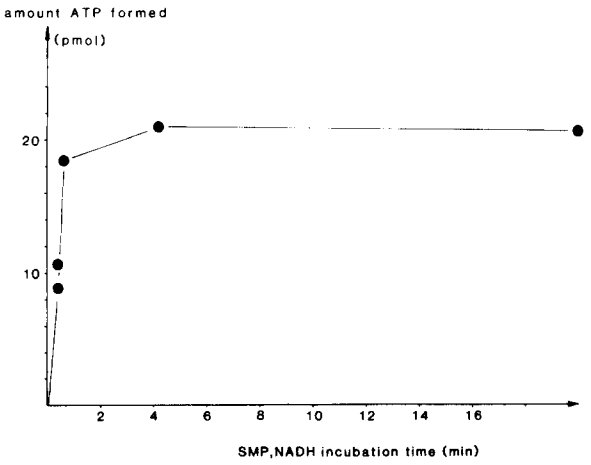


Fig. 3. The amount of ATP formed due to oligomycin stimulation is plotted against the time submitochondrial particles (SMP) (241 μg) have been preincubated with NADH (5 mM) in the assay mixture before the oligomycin addition (2 μg). Final volume: 0.5 ml. The luminometric method was used (see Materials and Methods).

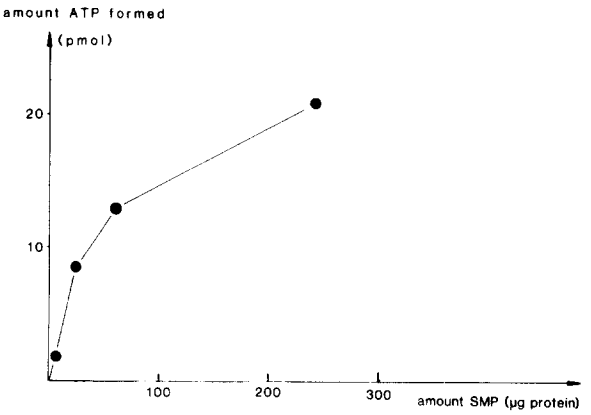


Fig. 4. Amount of ATP formed due to oligomycin stimulation after 10 min energization of submitochondrial particles (SMP) with NADH as a function of the amount of submitochondrial particles. Additions: 2 μg oligomycin and 5 mM NADH. Final volume: 0.5 ml. The luminometric method was used.

*Effect of oligomycin on free ATP formation with added ADP*

The effect of oligomycin on formation of free ATP with added ADP is dependent on the order of addition of NADH and ADP. In an experiment where submitochondrial particles were preincubated for 10 min with oligomycin and NADH was subsequently added, giving formation of free ATP with 'endogenous' ADP for about 10 min, the formation of free ATP upon subsequent addition of ADP was inhibited by the oligomycin by 60–80% (Fig. 5). If the preenergization of submitochondrial particles with NADH in the presence of oligomycin was performed in the absence of $P_i$ the inhibition of ATP formation from added $P_i$ and ADP was about 90% (Fig. 5). However, if ADP was added before NADH, oligomycin stimulated the formation of free ATP and the amount formed was proportional to the amount of ADP added.

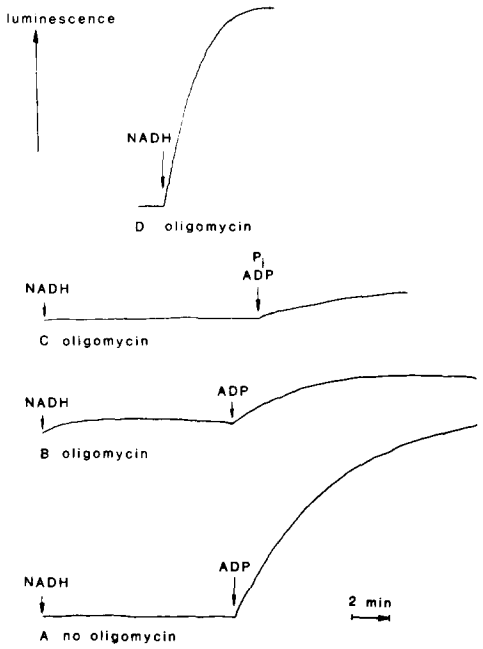


Fig. 5. Luminometric traces showing the effect of preenergization of submitochondrial particles with NADH in the presence of oligomycin on free ATP formation when exogenous ADP is added. NADH is added as indicated. (A) Energization in the absence of oligomycin. Submitochondrial particles were preincubated for 10 min in the absence of oligomycin (the buffer containing 3 mM $P_i$). About 3 pmol of ATP were released upon NADH addition (although not visible in the figure because a low sensitivity was chosen for studying the ATP formation due to ADP addition). Upon ADP addition about 55 pmol of ATP were formed. (B) Energization with oligomycin. Submitochondrial particles were preincubated in the presence of oligomycin for 10 min. About 4 pmol of ATP were formed upon NADH addition and 15 pmol upon ADP addition. (C) Energization with oligomycin in the absence of $P_i$. Submitochondrial particles were preincubated with oligomycin for 10 min in the absence of $P_i$. $P_i$ was added as indicated. No ATP was formed upon NADH addition before the addition of $P_i$ and ADP; this latter addition resulted in formation of 6.5 pmol ATP. (D) Submitochondrial particles were preincubated in the presence of oligomycin, $P_i$ and ADP for 10 min. Upon NADH addition 57 pmol of ATP were formed. In all experiments the final concentrations were: 40 $\mu$g/ml submitochondrial particles, 3 mM $P_i$, 5 $\mu$M NADH, 2 $\mu$g/ml oligomycin and 2 $\mu$M ADP.

*Adenine nucleotide content of submitochondrial particles*

Adenine nucleotide determinations which were made on submitochondrial particles show that treatment with NADH and oligomycin gave a decrease in ATP content which was 36, 33 and 35%, respectively, for three different preparations, whereas the effects on total adenine nucleotide content were quite variable (Table I). Although there are variations in the ATP content per unit protein, there is a consistent decrease upon treatment with oligomycin and NADH with roughly one of three ATP molecules.

TABLE I

NUCLEOTIDE DETERMINATIONS OF YEAST SUBMITOCHONDRIAL PARTICLES

Nucleotides are given as pmol/$\mu$g protein. NADH + oligo (in the preparation column) means that the particles have been treated with NADH and oligomycin in the same way as in the luminometric experiments, whereas 'control' means that no NADH or oligomycin has been added. See Materials and Methods for procedure. Only preparation (III) had $Mg^{2+}$ + ATP added during the preparation of the particles ('Mg-ATP particles') [19]. The absolute nucleotide content values should be higher than this table indicates [25], as losses of nucleotides may have occurred during extraction [26].

| Preparation | ATP | ADP | AMP |
|---|---|---|---|
| 850930 (I) | | | |
| Control | 0.070 | 0.005 | 0 |
| NADH + oligo | 0.045 | 0.022 | 0.023 |
| 851003 (II) | | | |
| Control | 0.140 | 0.022 | 0.023 |
| NADH + oligo | 0.094 | 0.055 | 0.016 |
| 851003 (III) | | | |
| Control | 0.200 | 0.003 | 0 |
| NADH + oligo | 0.130 | 0.014 | 0.009 |

## Discussion

*Absence of added ADP*

By the sensitive and continuous luciferin technique, we have been able to measure quantitatively events at very low concentrations of free ATP. This has given some entirely new and unexpected findings with oligomycin, which strongly stimulates the formation of free ATP over a wide range of oligomycin concentrations.

Our results show an NADH- and $P_i$-dependent stimulating effect of oligomycin on the formation of free ATP, when no ADP is added. Furthermore, our data indicate that oligomycin stimulates release of one-third of the tightly bound ATP. This suggests to us that the ATP release emerges

from one turnover of a single site in $F_1$ rather than from many turnovers resulting in partial release of ATP from three sites. The amount of free ATP formed is in the same order of magnitude as the amount of $F_1$ in ox-heart mitochondria (0.3–0.5 nmol/mg protein) [20].

Phosphate is believed to react directly, without a phosphoenzyme intermediate, with ADP in an in-line reaction, to form ATP [7]. Energy may be needed in the ATP release step rather than in the actual reaction step [8–10]. This could mean that in our experiments one molecule of ATP bound is formed from added $P_i$ and one tightly [6] bound ADP, and that this occurs at one of the three identical $\beta$-subunits. From another of the $\beta$-subunits a tightly bound ATP, already formed, may be released in an energy-requiring step. Oligomycin, which inhibits the utilization of the energy from the proton gradient, may either push the reaction:

$$ADP + P_i \rightleftharpoons ATP + H_2O$$

at one of the $\beta$-subunits to the right (without any addition of ADP to any other subunit) or facilitate the release of ATP, although no protons can be pumped through the enzyme, or both.

The nucleotide determinations, which show that treatment of submitochondrial particles with oligomycin and NADH in the presence of $P_i$ reduces the ATP content of submitochondrial particles, is in agreement with our conclusions. Approximately one-third of the ATP is released, which is similar to the observations of Penefsky [10] upon energization of ox-heart submitochondrial particles. Statistically, if the probability of ATP occupying one of the three different states of the $\beta$-subunits of the whole population of ATPase, is equal (i.e., 1/3 of each) and if ATP can be released only from one state, it would mean that 1/3 of the population of ATP molecules would be released. Our data are remarkably close to the theoretical 33.3%.

The oligomycin-stimulated hydrolysis and/or rebinding of the free ATP formed in the absence of added ADP could possibly be explained by the high $K_a$ value ($10^{12}$ $M^{-1}$) of binding of ATP to the enzyme [12]. Immediate rebinding and hydrolysis of released ATP was also recently observed by Penefsky [10].

*Presence of added ADP*

In the presence of added ADP the effect of oligomycin on the system is dependent on the order of addition of NADH and ADP (see Results). Only when submitochondrial particles were preenergized in the presence of oligomycin was an inhibition of formation of free ATP obtained. Oligomycin-stimulated formation of free ATP (release of ATP) during the preenergization does not seem to be the important step for enabling oligomycin to inhibit the ATP synthesis from added ADP, as inhibition was obtained even when $P_i$ was omitted during preenergization in the presence of oligomycin and thereby no free ATP formation occurred (Fig. 5). If ADP was added to submitochondrial particles already preincubated with oligomycin but without preenergization, oligomycin, instead of inhibition, showed stimulation of free ATP formation. It has earlier been observed that oligomycin effects may be dependent on the energy state of the membranes [21].

## Conclusions

Although it is tempting to believe that the stimulating effect of oligomycin is due to its tightening of leaky membranes by preferential binding of oligomycin to exposed $F_0$'s, this does not fully explain our results. Firstly, the same concentration of oligomycin as that stimulating formation of free ATP gives, after preincubation, inhibition of hydrolysis of added ATP, and secondly, neither high concentration of oligomycin (Fig. 2) nor preincubation of submitochondrial particles with oligomycin decreases its stimulating effect. Thus, an additional explanation to tightening of the membranes must be sought.

Reconstitution experiments with $F_0F_1$-ATPase from *Rhodospirillum rubrum* depleted of their $\beta$-subunits with $\beta$-subunits from *Escherichia coli* ATPase have indicated that the $\beta$-subunit of *R. rubrum* also plays an important role in conferring sensitivity to oligomycin [23]. Thus, our data in a particularly direct manner would appear to support the view that oligomycin may influence activities on the $\beta$-subunits in a more specific way than by only inhibiting proton translocation through $F_0$.

Finally, it has has now been possible to add a

substantial increase of information to our recently published general illustration of the differences in action between DCCD and oligomycin on the $H^+$-pyrophosphatase and $H^+$-ATPase of *R. rubrum* [24]. Lack of the $\beta$-subunit of the ATPase in the pyrophosphatase may well suffice as an explanation for the lack of inhibition of the pyrophosphatase by oligomycin.

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