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Phosphatase activity of H^+-ATPase from chloroplasts

Tânia Beatriz Creczynski-Pasa [a,1], Peter Gräber [b,*], Elias Walter Alves [a], André Teixeira Ferreira [a], Helena Maria Scofano [a]

[a] *Departamento de Bioquímica Médica, ICB / CCS, Universidade Federal do Rio de Janeiro, 21941-590 Rio de Janeiro, RJ, Brazil*
[b] *Institut für Physikalische Chemie, Albert-Ludwigs-Universität Freiburg, Albertstr. 23a, 79104 Freiburg, Germany*

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Abstract

The chloroplast H^+-ATPase (CF_0F_1) was purified from spinach chloroplasts and studied both in the soluble state and after reconstitution into asolectin liposomes. Hydrolysis of ATP and of *p*-nitrophenylphosphate (*p*-NPP) catalysed by CF_0F_1 was investigated. (1) Soluble, isolated CF_0F_1 catalyzes ATP hydrolysis and *p*-NPP hydrolysis. (2) ATP inhibits the phosphatase activity in the latent state ($K_i = 1.7$ mM). (3) Addition of 100 mM sulfite increases the rate of ATP hydrolysis by a factor of 10, while *p*-NPP hydrolysis is completely abolished. (4) CF_0F_1 reconstituted into asolectin vesicles catalyzes ATP hydrolysis and *p*-NPP hydrolysis. When the enzyme is brought into its active state by a $\Delta pH/\Delta\varphi$ jump, ATP hydrolysis is increased by a factor of 8, and *p*-NPP hydrolysis is completely abolished. (5) ATP hydrolysis by the activated enzyme is inhibited by *p*-NPP ($K_i = 1.6$ mM). (6) *p*-NPP also inhibits ATP synthesis by the activated enzyme, competing with phosphate ($K_i = 0.9$ mM). These results show that in the active state of CF_0F_1, *p*-NPP is not hydrolyzed but acts as a competitive inhibitor; in the inactive state of CF_0F_1, *p*-NPP is hydrolyzed. Hydrolysis of *p*-NPP might be used as an assay for the inactive forms of CF_0F_1.

Keywords: CF_0F_1; Chloroplast H^+-ATPase; *p*-Nitrophenylphosphate; Phosphatase

1. Introduction

Membrane-bound H^+-ATPases catalyze ATP synthesis and ATP hydrolysis coupled with transmembrane proton transport in bacteria, chloroplasts and mitochondria [1]. These enzymes have a hydrophilic part, F_1, containing six nucleotide binding sites and a membrane integrated part, F_0, which is involved in proton transport [2,3]. The F_1 part can be separated from the F_0 part and has been frequently used for functional and structural studies. Recently, the structure of a major portion of the F_1 part from mitochondria was reported with a resolution of 2.8 Å [4]. On the basis of the characteristics of its reaction cycle, the enzyme has been classified as an F-type ATPase [5].

The H^+-ATPase from chloroplasts has been isolated, purified and reconstituted with full ATP synthesis activity in liposomes [6–10]. ATP hydrolysis is

Abbreviations: *p*-NPP, *p*-nitrophenylphosphate; *p*-NP, *p*-nitrophenol; *p*NPPase, *p*-nitrophenylphosphatase; ATPase, adenosine 5′-triphosphatase; EDTA, ethylenediaminotetraacetic acid; TCA, trichloroacetic acid; CF_0F_1, H^+-ATPase from chloroplasts.

* Corresponding author. Fax: (49) (761) 203-61 89.

[1] Present address: Departamento de Ciências Fisiológicas, CCB, Universidade Federal de Santa Catarina, 88010-970 Florianópolis, SC, Brazil.

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catalyzed by the soluble CF_0F_1 in the latent state, at very low velocities. Following reconstitution into liposomes, the rate of ATP hydrolysis is increased after energization by a $\Delta pH/\Delta\varphi$ jump [11]. Unlike the F_1 parts from bacteria and mitochondria the isolated CF_1 does not catalyze ATP hydrolysis unless the enzyme is subjected to special treatment, e.g., heating, trypsination, reduction with dithiothreitol or addition of sulfite [11–14]. ATP hydrolysis catalyzed by CF_0F_1 requires Mg^{2+} as cofactor, whereas the specially treated CF_1 requires Ca^{2+}. It is clear that ATP hydrolysis by the F_1 parts is not coupled with proton transport, and therefore the mechanism might be different from that of the holoenzyme. The great majority of published reports on the F-type ATPases refer to the treated activated state of the enzyme, and the relationships on modifications in the conformations regarding the transitions form the latent to the activated state are poorly known.

In comparison with other ATPases, the F-type ATPases have been considered to be highly specific for nucleotides as substrates. So far, no information is available regarding the hydrolysis of non-nucleotide substrates by F-type ATPases. We now show that CF_0F_1 hydrolyzes *p*-NPP. We measure its kinetics and compare it with ATP hydrolysis catalyzed by the soluble enzyme and by the enzyme reconstituted into liposomes. We show that only CF_0F_1 in the latent state hydrolyzes *p*-NPP, and we suggest that hydrolysis of *p*-NPP may serve as a highly accurate probe for the catalytic site(s) of CF_0F_1 in the poorly known latent state.

2. Materials and methods

p-NPP (di-cyclohexylammonium salt), *p*-NPP (Mg salt), *p*-NP, Sephadex G-50 (medium) and ATP (Mg salt) were obtained from Sigma. $^{32}P_i$ was obtained from Brazilian Institute of Atomic Energy; [γ-^{32}P]ATP was prepared according to Maia et al. [15]. Asolectin was obtained from Fluka and used without further purification.

2.1. Isolation and reconstitution of CF_0F_1

CF_0F_1 ATPase was isolated from spinach chloroplasts according to Pick and Racker [6], as modified by Fromme et al. [8]. Routinely, the enzyme was stored in a concentration of 2–3 mg/ml in a buffer containing 30 mM Tris-succinate, pH 6.5, 2 mg/ml Triton X-100, 0.5 mM EDTA, 1 mg/ml asolectin and 900 mM sucrose, in the absence of ATP (which inhibits *p*-NPP hydrolysis). In order to test *p*-NPPase activity in other preparations containing ATP, the enzyme was eluted through centrifuge columns (Sephadex G-50, medium) [16]. Purified CF_0F_1 was incorporated into asolectin liposomes by cholate dialysis, as described earlier [7].

2.2. p-NPP hydrolysis

Latent CF_0F_1 (final concentration, 80 μg/ml) was added to a medium containing 2.5 mM $MgCl_2$, 50 mM KCl, 50 mM Tris-acetate at pH 7.0 or 8.0 and 3 mM *p*-NPP. Absorption of *p*-NP, the product of *p*-NPP hydrolysis, was measured at 415 nm over a period of 10 min in a recording spectrophotometer (Hitachi U-2000). The absorption coefficients for *p*-NP where ϵ (415 nm, pH 7.0) = 5700 $M^{-1} \cdot cm^{-1}$ and ϵ (415 nm, pH 8.0) = 12 000 $M^{-1} \cdot cm^{-1}$.

2.3. ATP hydrolysis by soluble enzyme

After pre-treatment with 50 mM dithiothreitol for 2 h, the ATPase activity was measured using 5 mM [γ-^{32}P]ATP, 0.39 mg/ml CF_0F_1, 5 mM $MgCl_2$, 50 mM Tris-HCl (pH 8.0) and sodium-sulfite when indicated. The reaction was stopped with 0.1 M HCl containing activated charcoal [17] to adsorb the [γ-^{32}P]ATP remaining from the reaction medium. The samples were centrifuged for 10 min at 2000 rpm and an aliquot of the supernatant containing $^{32}P_i$ was counted in a liquid scintillation counter.

2.4. ATP hydrolysis driven by $\Delta pH/\Delta\varphi$ jump

Liposomes reconstituted with CF_0F_1 were prepared by incubation in a buffer containing: $6.2 \cdot 10^{-8}$ M enzyme, 30 mM sodium succinate, 2 mM NaH_2PO_4, 0.6 mM KCl and 1 μM valinomycin, at pH 4.9. After 30 s pre-incubation, an equal volume of a second buffer containing 200 mM Na-Tricine, 120 mM KCl and 2 mM NaH_2PO_4 at pH 8.7, was added with continuous mixing (to give ΔpH 3.4). After 15 s the reaction was started with a mixture of 5 mM Mg-ATP

([γ-^{32}P]ATP 10^5 Bq/ml) and 10 mM NH_4Cl (final concentrations). Aliquots were stopped with 0.1 M HCl and activated charcoal. The samples were centrifuged and the $^{32}P_i$ was measured as described above.

2.5. ATP synthesis driven by $\Delta pH/\Delta\varphi$ jump

Proteoliposomes were treated as described above except that the concentration of NaH_2PO_4 was 5 mM in both buffers and after the second buffer 200 μM ADP instead of ATP was added to start the reaction. In this case the NH_4Cl was not added. The reaction was interrupted with trichloroacetic acid to a final concentration of 2% (w/v). ATP produced was measured by the luciferin-luciferase method [7].

2.6. p-NPP hydrolysis following a $\Delta pH/\Delta\varphi$ jump

p-NPPase activity was measured as described for ATP synthesis except that phosphate was omitted in both buffers and that *p*-NPP (in this case, the Mg salt) was added after addition of the second buffer, either together with 10 mM NH_4Cl, or without NH_4Cl. Blanks without energization were prepared by mixing the two buffers before adding the proteoliposomes. The *p*-NP produced was measured as described above.

3. Results

3.1. Characterization of p-NPP hydrolysis by soluble CF_0F_1

Incubated with a high concentration of *p*-NPP (3 mM), purified enzyme in the soluble, latent state hydrolyzes *p*-NPP at low rates. The *p*-NPP hydrolysis is linear up to 10 min and its rate is calculated from the slope. As expected for an enzyme-catalyzed reaction, the rate of *p*-NPP hydrolysis, increases linearly with the protein concentration up to 100 μg/ml (not shown). The phosphatase activity was not altered by pre-treatment with reducing reagents (e.g., 50 mM dithiothreitol for 2 h) (not shown).

Hydrolysis of *p*-NPP can be described by Michaelis–Menten kinetics, and it varies with the pH. At pH 7.0, CF_0F_1 displays a K_m for *p*-NPP of 1.6 mM and a higher rate ($V_{max} = 0.85$ μmol *p*-NP · $mg^{-1} \cdot h^{-1}$) than at pH 8.0 ($K_m = 2.26$ mM and $V_{max} = 0.26$ μmol *p*-NP · $mg^{-1} \cdot h^{-1}$) (Fig. 1). This result indicates that the *p*-NPPase activity is stimulated by H^+. The rate of *p*-NPP hydrolysis by the latent enzyme is only a small fraction (0.4% at pH 7.0 and 0.14% at pH 8.0) of the maximal rate of ATP hydrolysis attainable in our preparations.

3.2. Reciprocal inhibition of p-NPP and ATP hydrolysis

ATP was found to be an inhibitor of *p*-NPP hydrolysis by soluble CF_0F_1. The degree of inhibition depends on the *p*-NPP concentration (Fig. 2A). A Dixon plot (inset) shows a competitive inhibition by ATP, with a K_i of 1.7 mM.

The soluble CF_0F_1 was reconstituted into asolectin vesicles and the vesicles were energized by establishment of a transmembrane electrochemical gradient. In the presence of 5 mM ATP the velocity of ATP hydrolysis after energization reached 30 mol ATP · mol $CF_0F_1^{-1} \cdot s^{-1}$, which is comparable to values

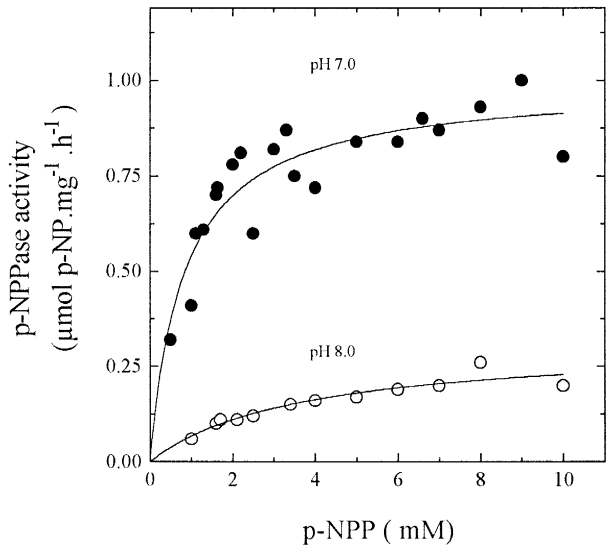

Fig. 1. *p*-NPP dependence of *p*-NPP hydrolysis by soluble CF_0F_1. The assay medium contained 50 mM Tris-acetate, 3 mM $MgCl_2$, 50 mM KCl, 80 μg/ml CF_0F_1 at 25° and *p*-NPP as indicated. At pH 7.0 (filled circles), $K_m = 1.16 \pm 0.07$ mM and $V_{max} = 0.85 \pm 0.06$ μmol *p*-NP · $mg^{-1} \cdot h^{-1}$. At pH 8.0 (open circles), $K_m = 2.26 \pm 0.01$ mM and $V_{max} = 0.26 \pm 0.01$ μmol *p*-NP · $mg^{-1} \cdot h^{-1}$. The kinetic parameters were obtained using the program Enzfitter.

reported elsewhere [8]. Addition of increasing amounts of *p*-NPP leads to an inhibition of the ATPase activity (Fig. 2B). The Dixon plot at a fixed ATP and variable *p*-NPP concentrations indicates competitive inhibition of ATP hydrolysis by *p*-NPP ($K_i = 1.6$ mM).

3.3. Effect of activation by $\Delta pH / \Delta\varphi$ on the ATPase and phosphatase activities of CF_0F_1

In accordance with a previous report [7] the rate of ATP hydrolysis is unaffected by incorporation of the enzymes into liposomes, and it increases approximately 8-fold following a $\Delta pH/\Delta\varphi$ jump (Table 1). In marked contrast with the effect on ATPase activity, incorporation of CF_0F_1 into asolectin vesicles increases the rate of *p*-NPP hydrolysis approximately 20-fold. Energization completely abolishes the *p*-NPPase activity (Table 1). Usually the ATPase activity is expressed in mol P_i per mol CF_0F_1 per s (see Fig. 2B). In Table 1 both the ATPase and the phosphatase activities are expressed in μmol/mg/h in order to facilitate comparison.

3.4. Effect of sulfite on ATPase and phosphatase activities

Sulfite has been reported to stimulate the ATPase activity of thylakoid membranes [13] and CF_1 [14]. Although the effect of this anion is not completely understood, it has been proposed that sulfite, like the $\Delta pH/\Delta\varphi$ jump, promotes the release of tightly bound ADP from the enzyme [13,14]. Here we show that it is also possible to activate ATP hydrolysis by purified soluble CF_0F_1 with sulfite (Fig. 3 inset) and to reach rates in the same order of magnitude to those attained by a $\Delta pH/\Delta\varphi$ jump of Table 1 and Fig. 2. However, the *p*-NPPase activity is strongly inhibited by sulfite, with a half maximal effect at 0.1 mM, much lower than required to activate ATP hydrolysis (10 mM) (Fig. 3).

Fig. 3 also shows that phosphate inhibits *p*-NPP hydrolysis, with a profile distinct from that of sulfite. The concentration dependence for inhibition by sulfite can be fitted by two components; this observation may mean that sulfite binds at two different sites, with different affinities. Phosphate inhibition exhibits

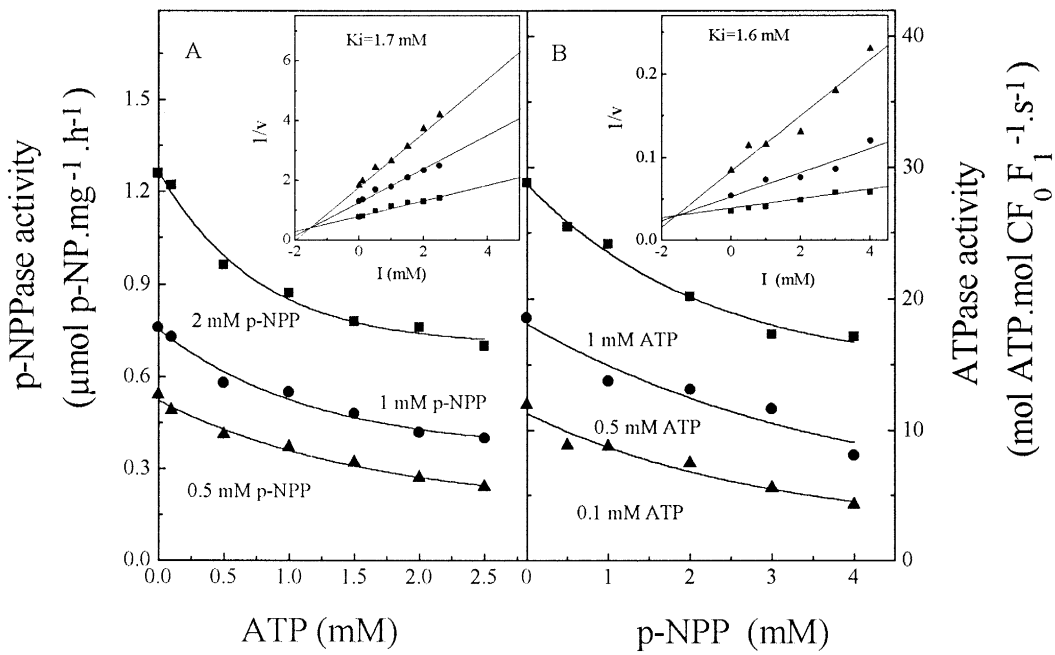

Fig. 2. Effect of ATP on *p*-NPP hydrolysis by soluble CF_0F_1 and effect of *p*-NPP on ATP hydrolysis by reconstituted CF_0F_1 driven by $\Delta pH/\Delta\varphi$ jump. A: the assay medium contained 50 mM Tris-acetate, 3 mM $MgCl_2$, 50 mM KCl, at pH 7.0, and the ATP and *p*-NPP concentrations as indicated. Inset: data replotted as a Dixon plot; B: the conditions for measuring ATP hydrolysis are described in Section 2. *p*-NPP and [γ-^{32}P]ATP concentrations as indicated. Inset: replot of the data as a Dixon plot.

Table 1
Rates of ATP hydrolysis and *p*-NPP hydrolysis by soluble and reconstituted CF_0F_1

Conditions	*p*-NPPase activity (μmol *p*-NP $\cdot$ mg^{-1} $\cdot$ h^{-1})		ATPase activity (μmol Pi $\cdot$ mg^{-1} $\cdot$ h^{-1})	
	soluble	reconstituted	soluble	reconstituted
$-\Delta pH/\Delta\phi$ jump	0.26 ± 0.01	5.5 ± 2.0	28 ± 2.0	28 ± 2.0
$+\Delta pH/\Delta\phi$ jump	–	0.0 [a]	–	200 ± 20

For the hydrolytic activity, reconstituted enzyme was energized by a $\Delta pH/\Delta\phi$ jump in the absence of phosphate. Fifteen seconds later the proteoliposomes were added to the assay medium for ATP hydrolysis or *p*-NPP hydrolysis. ATP hydrolysis was measured in medium containing 5 mM $MgCl_2$, 50 mM Tris-HCl pH 8.0, 5 mM [γ-^{32}P]-ATP and 0.39 mg/ml CF_0F_1. For *p*-NPP hydrolysis the assay medium contained 50 mM Tris-acetate pH 8.0, 3 mM $MgCl_2$, 50 mM KCl, 3 mM *p*-NPP and 80 μg/ml CF_0F_1.
[a] No difference from the base line.

only a single component. Half maximal inhibition was observed at 0.9 mM phosphate. These observations might be useful for further studies of substrate binding on CF_0F_1.

3.5. Effect of p-NPP on ATP synthesis

On the basis of the results of Table 1 and Fig. 3, it can be suggested that either *p*-NPP does not bind to the activated state of the enzyme, or that it binds to the activated state and is not hydrolyzed. It is hydrolyzed exclusively by the enzyme in the latent state. In order to help to discriminate between these possibilities, the reconstituted enzyme was activated by a $\Delta pH/\Delta\varphi$ jump as described in Section 2, and the ATP synthesis was measured at three different concentrations of P_i, varying the concentration of *p*-NPP. Fig. 4 shows competitive inhibition between *p*-NPP and P_i ($K_i = 0.9$ mM) for ATP synthesis by activated CF_0F_1. This result is in line with the data of Fig. 3, which shows inhibition of *p*-NPP hydrolysis by phosphate in the latent state, and raises the possibility

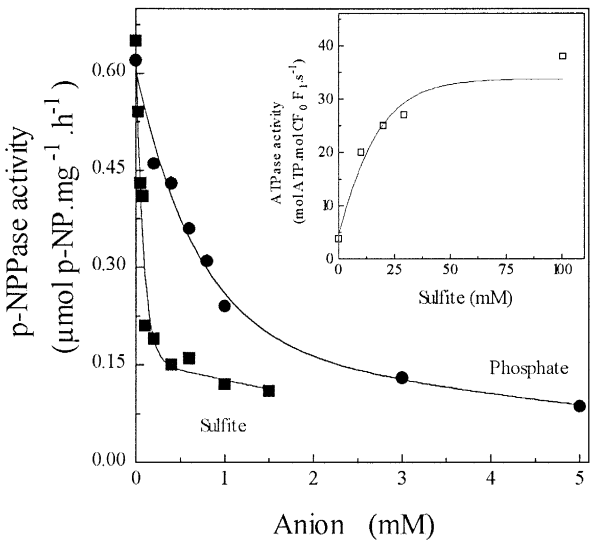

Fig. 3. Comparison of sulfite and phosphate effects on the phosphatase and ATPase activities. The assay medium for phosphatase activity was the same, as that described in Fig. 1 at pH 7.0, with a *p*-NPP concentration of 3 mM. The ATPase activity (inset) was measured as described in the legend of Table 1, with soluble enzyme. P_i and sulfite were present as indicated.

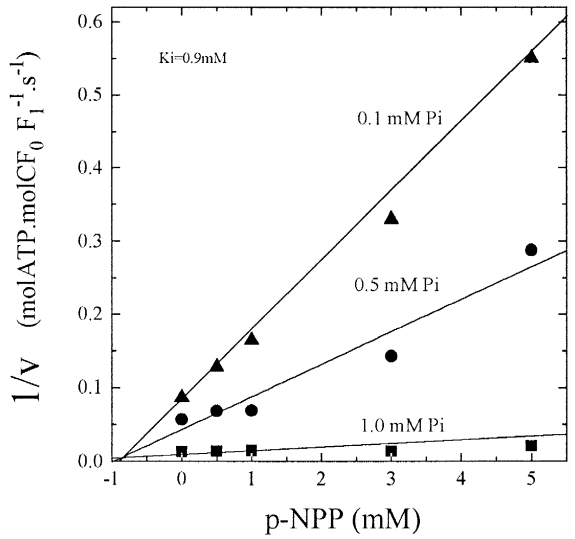

Fig. 4. Effect of *p*-NPP on ATP synthesis by CF_0F_1 reconstituted into asolectin vesicles. Enzyme ($6.2 \cdot 10^{-8}$ M) was incubated for 30 s in a buffer containing: 30 mM Na-succinate pH 4.9, NaH_2PO_4 (1 mM, 0.5 mM or 0.1 mM, as indicated in the figure), 0.60 mM KCl and valinomycin 1 μM (freshly added). After this time a $\Delta pH/\Delta\varphi$ jump was performed with a second buffer containing sodium tricine 200 mM, NaH_2PO_4 (1 mM, 0.5 mM or 0.1 mM), 120 mM KCl and 200 μM ADP (freshly added). The reaction was stopped by adding TCA to a final concentration of 2%. The ATP concentration was determined by the luciferin-luciferase method (see Section 2).

that there are common or mutually exclusive binding sites for *p*-NPP and either P_i or ATP, both in the latent and in the activated state of the enzyme. Comparison of the K_i for inhibition of ATP synthesis by *p*-NPP obtained in Fig. 4 with the K_m for *p*-NPP hydrolysis observed in Fig. 1, suggests that the compound binds to both the latent and the activated state of the enzyme with similar affinities, but that only the latent state is catalytically competent. It should also be noted that the Mg-*p*-NPP salt used in this experiment does not uncouple the pH gradient during ATP synthesis, because the activity was not influenced by 3 mM Mg-*p*-NPP when 5 mM P_i was present (not shown).

4. Discussion

In common with other F-type ATPases, CF_0F_1 has a complex structure, and the mechanisms involved in hydrolysis and synthesis of nucleotides are not well understood. In comparison with other phosphohydrolytic enzymes, the enzymes that couple ATP hydrolysis to H^+ transport have been reported to be highly specific for nucleotides as substrate. The F_0F_1 from *Escherichia coli* also displays GTP synthesis and hydrolysis that are not regulated by the binding of adenine nucleotides at non-catalytic sites [18]. Bovine heart submitochondrial particles containing F_0F_1 hydrolyze ITP and generate a proton gradient at the expense of ITP hydrolysis, although the enzyme does not synthesize ITP [19]. CF_1 catalyzes GTP hydrolysis, an activity that depends on the occupancy of non-catalytic sites [20] and is markedly inhibited by low concentrations of ADP [21]. ITP and CTP also bind to the β-subunit of CF_1 [22]. ATP synthesis by CF_0F_1 was shown with several ribose and base modified nucleotides and thiophosphate analogs [23–25].

Up to now, there are no reports on hydrolysis of non-nucleotide substrates by F-type ATPases. A photoreactive isomer, 4-azido-2-nitrophenyl phosphate, ANPP, has been used as a phosphate analogue for studying the phosphate binding site. ANPP is not hydrolyzed by the F_1 from mitochondria [26], *E. coli* [27] and chloroplasts [28]. The compounds β-naphthyl di, tri, or tetraphosphate were reported to inhibit ATPase activity of isolated CF_1 and light-triggered ATPase activity of chloroplasts, competing with ATP. None of these compounds, however, was hydrolyzed by these preparations. This observation suggests that they bind at the catalytic site, but are not catalysed by the enzyme [29].

This report shows that in contrast to ANPP or the β-naphthyl derivatives, the latent CF_0F_1 hydrolyzes *p*-NPP at low rate, and describes conditions that favor hydrolysis. A striking reciprocal inhibition is observed when either substrate is present during hydrolysis of the other (Fig. 2). In addition, *p*-NPP competes with P_i as an inhibitor of ATP synthesis by activated CF_0F_1 (Fig. 4). We conclude that *p*-NPP binds to the catalytic site in both active and latent conformations, presumably at the site where P_i binds during ATP synthesis. It is noteworthy that purified soluble CF_1, lacking F_0 and obtained by a completely different technique, is also able to hydrolyze *p*-NPP (not shown).

The *p*-NPPase activity of soluble CF_0F_1 described in this paper is low, compared with the ATPase activity of the energized enzyme (about 0.4% at pH 7.0). However, it is comparable to *p*-NPP hydrolysis by P-type ATPases, e.g., the H^+-ATPase from *Schizosaccharomyces pombe* [30]. It is interesting that *p*-NPP hydrolysis by CF_0F_1 depends strongly on the conformational state: only the latent enzyme forms, i.e. the soluble CF_0F_1 and the reconstituted CF_0F_1 not activated by a $\Delta pH/\Delta\varphi$ jump, hydrolyze *p*-NPP. It is intriguing that *p*-NPP hydrolysis is highly activated after enzyme incorporation into liposomes (Table 1). This clearly indicates that the conformation of latent CF_0F_1 is changed when it accommodates to the lipid environment, although this change is not reflected in ATP hydrolysis.

This suggests that *p*-NPP hydrolysis may provide a more sensitive parameter for the study of the catalytic site in the latent state than ATP hydrolysis, and indicates that the enzyme in the latent state is highly sensitive to the hydrophobic environment.

Hydrolysis of *p*-NPP has been described for a number of ATP hydrolytic enzymes and extensively studied in the case of P-type ATPases such as Na^+-K^+-ATPase (for reviews, see [31] and [32]), Ca^{2+}-ATPase from sarcoplasmic reticulum [33], H^+-ATPase form *Schizosaccharomyces pombe* [30] and Ca^{2+}-ATPase from plasma membranes [34,35]. These enzymes exhibit an E_1–E_2 hydrolytic cycle that couples ATP hydrolysis with transmembrane ion trans-

port. The E_1 form binds both ATP and the ion to be transported with high affinity, forming a phosphoenzyme intermediate $E_1^{ion} \sim P$. The $E_2^{ion} \sim P$ form is generated subsequently to the ion transport, with a concomitant change in the conformation of the enzyme active site. Also in the case of P-type ATPases, *p*-NPP hydrolysis is highly sensitive to the active site conformation and has been widely used as a tool to study the E_2 form of the enzymatic cycle. With the exception of sarcoplasmic reticulum [36] hydrolysis of *p*-NPP is catalyzed exclusively by the uncoupled ('inactive' for ATP hydrolysis) E_2 form.

On the basis of our data, it is clear that *p*-NPP has potential as a probe for different conformational states of F-type ATPases, as has already been shown for the several P-type ATPases: *p*-NPP hydrolysis provides a simple, functional and highly sensitive assay for the latent forms of CF_0F_1.

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