The mechanism of inhibition of yeast mitochondrial F1-ATPase by its natural regulatory peptide, IF1, was investigated by correlating the rate of inhibition by IF1 with the nucleotide occupancy of the catalytic sites. Nucleotide occupancy of the catalytic sites was probed by fluorescence quenching of a tryptophan, which was engineered in the catalytic site ($\beta$-Tyr345Trp). Fluorescence quenching of a $\beta$-Trp345 indicates that the binding of MgADP to F1 can be described as 3 binding sites with dissociation constants of $K_d1=10\pm2$ nM, $K_d2=0.22\pm0.03$ µM, $K_d3=16.3\pm0.2$ µM. In addition, the ATPase activity of the $\beta$-Trp345 enzyme followed simple Michaelis-Menten kinetics with a corresponding $K_m$ of 55 µM. Values for the $K_d$ for MgATP were estimated and indicate that the $K_m$ (55 µM) for ATP hydrolysis corresponds to filling the third catalytic site on F1. IF1 binds very slowly to F1-ATPase depleted of nucleotides and under unisite conditions. The rate of inhibition by IF1 increased with increasing concentration of MgATP to about 50 µM, but decreased thereafter. The rate of inhibition was half-maximal at 5 µM MgATP, which is 10 fold lower than the $K_m$ for ATPase. The variations of the rate of IF1 binding are related to changes in the conformation of the IF1 binding site during the catalytic reaction cycle of ATP hydrolysis. A model is proposed that suggests that IF1 binds rapidly, but loosely to F1 with two or three catalytic sites filled, and is then locked in the enzyme during catalytic hydrolysis of ATP.

$F_0F_1^{-1}$ proton ATPases (or ATP synthases), present in energy-transducing membranes, are molecular motors powered by a protonotive force generated by an electron transport chain (1). ATP synthases catalyze the phosphorylation of ADP forming ATP. The ATP synthase consists of two subcomplexes: $F_0$ and $F_1$. $F_0$ is an integral membrane protein complex minimally composed of $a_b c_{10}$ and it acts as a proton-driven turbine (2). The extrinsic $F_1$ moiety is composed of five different subunits with the stoichiometry $a_3 b_3 c$. $F_1$ contains three catalytic and three non-catalytic nucleotide-binding sites, located at $\alpha/\beta$ interfaces (3-4). $F_1$ and $F_0$ are connected by a central stalk composed of the $\gamma$, $\delta$, and $\epsilon$ subunits.

In the mitochondrion, movement of protons from the cytosol to the matrix drives the $F_0$ turbine, which rotates the central stalk. There is a second or peripheral stalk, which holds together the static parts of the machine including the core $F_1$ unit. Rotation of the central stalk in $F_1$ sequentially modifies the interactions of the $\gamma$-subunit with each of the catalytic sites thereby altering the nucleotide affinity of each of the three catalytic sites. Thus, $F_1$ has 3 catalytic sites each with different catalytic properties including their affinity for nucleotides. $F_1$ couples ATP hydrolysis to the rotation of the $\gamma$ subunit in the opposite direction of that observed during ATP synthesis (5-6).
Single-molecule studies indicated that hydrolysis of ATP is coupled with 120° rotation of the γ-subunit within the core of the ATPase. This 120° step was subdivided into two substeps of 80-90° which is related to the binding of ATP and 30-40° which is related to the hydrolysis of ATP or the release of Pi (7,8). Despite these and other studies, there are still debates on key features of the catalytic cycle. For instance, some reports conclude that a significant rate of ATP hydrolysis only occurs when each of the catalytic sites is filled with nucleotide (trisite catalysis) (6, 8-9), whereas others suggest that maximal ATP hydrolysis and synthesis occurs with just 2 nucleotide binding sites filled (bisite catalysis) (7, 10-11). Also, a detailed understanding of the cooperative aspects of the catalytic mechanism is still lacking.

The mitochondrial FoF1 ATP synthase is regulated in the mitochondrion. The enzyme is inhibited by the absence of protonmotive force and the presence of ATP, and it is activated by the presence of a protomotive force. This regulation, which is thought to avoid futile ATP hydrolysis, occurs by an inhibitory peptide, IF1 (review in ref. 12). IF1 exists in animal (13), plant (14) and yeast (15) mitochondria. IF1 is a small peptide, with well-conserved sequence: 84 residues in animals and 63 residues in yeast. IF1 binds to the F1 sector of the ATP synthase and also to mitochondrial F1 freed from the ATP synthase. Generation of a protonmotive force across the mitochondrial membrane releases IF1 from the ATP synthase (12, 16-22), or, alternatively, shifts this regulatory peptide from an inhibitory to a silent position on the enzyme (23-25). IF1 was proposed to bind to β-subunit (26-27), the α/β interface (28), and the α/γ interface (29). More recently, the crystal of bovine IF1-F1 complex was solved and it showed IF1 bound at the α/β catalytic interface with weak interactions with the γ subunit (30).

The crystal structure is a "snap-shot" in the dynamic mechanism of the binding and inhibition by IF1. ATP hydrolysis is necessary for IF1 binding (31), and there is a complex relationship between ATP concentration and rate of IF1 binding to isolated bovine F1-ATPase (32). IF1 binding rate increased at submicromolar ATP concentrations, reaching a maximum at concentrations of MgATP from 20-100 µM, but this rate dramatically decreased when MgATP concentrations reached 1-100 mM. Assuming bisite mode of ATP hydrolysis, it was proposed that IF1 could only bind F1 molecules containing one loosely bound ADP molecule and one ATP molecule. The decrease in the rate of IF1 binding at the higher MgATP concentration was suggested to be due to the transition from bisite to trisite catalysis (32). However, these proposals remained speculative, due to the absence of measurement of nucleotide occupancy of the catalytic sites.

In E. coli F1-ATPase, nucleotide binding in the catalytic pocket could be probed by the fluorescence quenching of a tryptophan replacing a tyrosine in the active site of the enzyme, β-Y331W (9). The corresponding replacement has been made in the yeast F1 ATPase, β-Y345W, and this enzyme has been used to measure the relationship of nucleotide site occupancy with ATPase activity and the rate of IF1 binding and inhibition. The results from this study provide three unique dissociation constants for MgADP binding and indicate that trisite catalysis is the predominant mode of ATP hydrolysis. Furthermore, IF1 binds to F1 at a similar and high rate when it has two or three nucleotide-filled catalytic sites. However, IF1 binds to F1 very slowly if it is nucleotide depleted or if just a single catalytic site is filled with nucleotide. A model is proposed where the rate of IF1 binding to F1 is controlled by changes in the conformation of the IF1 binding site on F1, which depends on the catalytic state of the enzyme. Once bound, IF1 is rapidly locked in place by the hydrolysis of ATP.

**Experimental Procedures**

**Yeast Strains and growth conditions** - Saccharomyces cerevisiae cells (Euroscarf BY4741-ΔATP2 MAT a, his3Δ1, leu2Δ0, met15Δ0, ura3Δ0, atp2::G418) were transformed with the plasmid pRS344SB (ATP2-Y345W) (33) as described in (34). Transformed cells were selected on a strictly respiratory medium containing 1% yeast extract, 1% bactopeptone and 3% glycerol (28°C). Transformed cells and WT cells (Euroscarf BY4741 MAT a, his3Δ1, leu2Δ0, met15Δ0, ura3Δ0) were grown at 28°C in a large volume...
of strictly respiratory medium.

**Purification of nucleotide-depleted F$_1$-ATPase** - Yeast F$_1$-ATPase was purified using prior method (35) with the following modifications. Chloroform extraction and precipitation with 70% saturated SO$_4$(NH$_4$)$_2$ were followed by gel exclusion chromatography using Sephadex G-25 (15 mL column) equilibrated with a buffer containing 50% glycerol (v/v), 5 mM EDTA and 100 mM Tris-SO$_4$, pH 7.5 (TEG). The active fractions were eluted with TEG and directly submitted to HPLC using TSK Spherogel SW4000 (Beckman Coulter Inc., Fullerton, CA, USA) column (0.75 x 30 cm) at an elution rate of 0.3 mL/min with TEG. The fractions containing F$_1$ were pooled and dialyzed for 2 hours at room temperature against 20 mM Tris-SO$_4$, pH 7.5, 1 mM EDTA (TE). The sample was precipitated with 70% saturated SO$_4$(NH$_4$)$_2$, centrifuged and dissolved in a minimal volume of TE containing 70% saturated SO$_4$(NH$_4$)$_2$. The nucleotide (ATP and ADP) content of the enzyme was estimated, after denaturation, to less than 0.01 mol/mol using a luciferase assay (36) where ADP was converted to ATP with phosphoenolpyruvate and pyruvate kinase. The rate of ATP hydrolysis was measured at pH 8.4, 30°C, in presence of 4 mM MgATP and 40 mM K-bicarbonate. The turnover number for ATP hydrolysis was 440 s$^{-1}$ for the wild type F$_1$-ATPase and 360 s$^{-1}$ for β-Y345W F$_1$-ATPase, based on a molecular mass of 372 kDa.

**Overexpression and purification of IF1** - The gene encoding IF1 (INH1) gene was amplified by polymerase chain reaction (PCR) from genomic yeast DNA kindly provided by Dr Jean Velours (Bordeaux, France). The PCR fragment was ligated into plasmid pET-30a(+) and transformed into *E. coli* BL21 (DE3) by electroporation using the apparatus and the procedure from Bio-Rad (Hercules, CA, USA). IF1 was overexpressed in LB medium after incubation with 0.5 mM isopropyl-β-D-thiogalactopyranoside (after the O.D. at 600 nm of the cell culture reached 0.6) for 3 hours at 37°C. The expressed IF1 had a N-terminal (His)$_6$-tagged extension and an enterokinase-mediated proteolytic cleavage site. For purification of IF1, bacteria were incubated with lysozyme (0.5 mg/mL) for 1 hour at room temperature and briefly sonicated on ice and in the presence of 1 mM phenyl methyl sulfonyl fluoride, 10 µg/mL RNase and 10 µg/mL DNase. The lysate was centrifuged at 25,000 g for 20 min (10°C) and the supernatant was incubated, under gentle stirring, with Ni-NTA resin (3 mL, Qiagen GmbH, Hilden, Germany) prepared in binding buffer (BB), 10 mM imidazole, 0.3 mM NaCl, and 50 mM Na$_2$HPO$_4$, pH 8. The resin was poured into a 0.9 cm diameter column and the column was washed sequentially with 10 and 4 volumes of the BB containing imidazole at 10 mM and 20 mM, respectively. IF1 was eluted by gravity with 2 volumes of BB containing 250 mM imidazole. The fractions containing IF1 were pooled and dialyzed for 7 hours at 4°C against a buffer containing 20 mM Tris, 50 mM NaCl, and 2 mM CaCl$_2$, pH 7.4. The (His)$_6$-tag was removed by digestion with enterokinase (12.5 U/mL) overnight at 18°C and enzymatic cleavage was stopped by heating the sample at 90°C for 10 min. The sample was centrifuged in a benchtop centrifuge for 10 min at 10°C, and the supernatant was eluted on a 0.9 cm diameter column containing Ni-NTA resin (2 mL) using the BB containing 10 mM imidazole. IF1 that did not bind to the column was collected, precipitated on ice with 10% trichloroacetic acid and collected with benchtop centrifugation. The pellet was dissolved in water at a final protein concentration of 5-10 mg/mL and stored at -80°C. IF1 appeared as a single band on SDS-PAGE. Due to the presence of the N-terminal enterokinase cleavage site, the first three amino acids of the recombinant and processed enzyme were Ala-Met-Ala, as compared to the natural IF1, which starts with Ser. However, the inhibitory activity of the recombinant IF1, as measured by the $k_{on}$ rate constant of formation of the IF1-MF$_1$ complex in the presence of 1 mM MgATP, was the same as the natural IF1 from yeast or a chemically synthesized product made by Neosystem (Strasbourg, France) (37).

**Protein titration** - Protein concentration was determined using micro-BCA titration kit (Pierce Chemical Co., Rockford, IL, USA).

**Fluorescence measurements** - Fluorescence experiments were carried out in a SFM25 fluorimeter (Kontron Instruments, Saint-Quentin-en-Yvelines, France) with excitation at 295 nm (bandwidth 5 nm) and emission at 350 nm (bandwidth 5 nm). The cuvette, stirred and
thermostatted at 25°C, was filled with buffer containing 50 mM MES, 20 mM KCl and 1 mM MgCl₂, pH 6.5 (2 mL). After measuring the background signal, the fluorescence of the purified F₁-ATPase (final concentration: 5 to 30 nM) was measured. MgATP, MgADP, or MgAMP-PNP (final concentration: 20 nM to 8 mM) was added after 30 s with rapid mixing as a small and constant volume of concentrated (5 µM-1 M) solution. The steady state fluorescence level was reached within 1 min. The F₁-ATPase retained full enzymatic activity for more than 2 min under these conditions. Fluorescence values were corrected for the fluorescence of nucleotides, for the dilution effects, and for the internal filter effect of the nucleotides. The inner filter effect was estimated from the nucleotide-induced decrease of fluorescence of chloroplast F₁-ATPase or wild type yeast F₁-ATPase. A unique correction curve was obtained for each of the nucleotides used (ADP, ATP, AMP-PNP), for all F₁ species and for different F₁-ATPase concentrations.

**ATP hydrolysis measurement and kinetic analysis** - Continuous monitoring of ATP hydrolysis coupled to NADH oxidation was carried out spectrophotometrically as described (37). The reaction was in a stirred and thermostatted cuvette (25°C) containing 50 mM MES pH 6.5, 20 mM KCl, 1 mM MgCl₂, 1 mM phosphoenolpyruvate (PEP), 20 units/mL pyruvate kinase (PK), 50 units/mL lactate dehydrogenase (LDH), 0.4 mM NADH and MgATP at from 0.5 µM to 20 mM. The ATPase reaction was initiated by adding F₁-ATPase at the indicated final concentration (1-10 nM) and measured as a decrease in absorbance of NADH at 340 nm. When a constant rate of ATP hydrolysis was reached, IF1 (75 nM) was injected and ATPase activity decayed. The spectrophotometric recording was analyzed as in (37), using the equation:

\[y(t) = V t + \left[\frac{(V_0 - V)}{k_{app}}\right](1 - e^{-k_{app}t}) + y_0\]  
Eqn. (1),

were \(y_0\) and \(y(t)\) are \(A_{340}\) at zero time and t time after IF1 addition, respectively. \(V_0\) the initial rate of \(A_{340}\) change (at zero time), \(V\) the final rate of \(A_{340}\) change (at infinite time), and \(k_{app}\) the apparent deactivation rate constant. ATP hydrolysis in the absence of regenerating system was measured in a small vessel, stirred, thermostatted at 25°C, containing the ATPase reaction medium devoid of PK, LDH and NADH. The ATPase reaction was stopped by addition of IF1 (5 µM). An aliquot (80 µL) was taken and added to spectrophotometric cuvette containing the complete reaction medium (1 mL) supplemented with 1 µM IF1. The level of ADP was determined from the absorbance decrease at 340 nm, with corrections for any dilution effects, and for the low level of ADP and pyruvate initially present in the reaction medium.

**Data analysis** - Fluorescence and kinetic data were fitted to non-linear theoretical models by iteration, using Excel (Microsoft Corporation, Redmond, WA, USA). Parameters to determine were defined as variables and their values were taken after the target cell, containing the average quadratic variation between the theoretical curve and the experimental data, converged to its minimum value.

**Chemicals and reagents** - All reagents were of analytical grade. Nucleotides were obtained from Roche (Basel, Switzerland). Yeast extract and Bactopeptone were from Difco (Detroit, MI, USA). DNA amplification by PCR was performed using Red Taq Polymerase from Sigma Chemical Co. (St. Louis, MO, USA). Plasmid purification used the Agarose DNA Extraction Kit from Roche (Basel, Switzerland). Calf intestinal phosphatase used for the dephosphorylation of pET 30a(+) was from Amersham Biosciences Europe GmbH (Freiburg, Germany). DNA ligation was carried out using the Fast-Link DNA Ligation Kit from Epicentre Technologies (Madison, WI, USA). DNA was prepared using the kit obtained from Qiagen (Qiagen GmbH, Hilden, Germany).

**RESULTS**

**Nucleotide affinity for each of the three catalytic sites** - The F₁-ATPase has three catalytic sites, which have been demonstrated to have different affinities for adenine nucleotides (9). We measured nucleotide affinity for each of the catalytic sites in order to relate catalytic site occupancy with the rate of IF1 binding to F₁. Catalytic site occupancy was measured by the fluorescence quenching of a tryptophan residue that replaced \(\beta\)-Tyr345, which is at the active site of the yeast F₁-ATPase. This method is...
analogous to that used by Senior's laboratory to probe nucleotide binding to catalytic sites of the *E. coli* F₇-ATPase (9) and is validated by the crystal structure of yeast mitochondrial F₇-ATPase which shows aromatic stacking of β-Tyr345 and the purine ring of the adenine nucleotide (4).

Figure 1a shows the fluorescence response of a nucleotide-depleted preparation of β-Y345W F₇-ATPase to addition of nucleotide. The addition of MgAMP-PNP (1 µM) does not affect wild type F₇-ATPase fluorescence (curve 1) but decreases the fluorescence of β-Y345W F₇-ATPase (curve 2). Curves 3-5 show kinetics of binding MgADP, at three different nucleotide concentrations, to nucleotide-depleted β-Y345W F₇-ATPase as measured by the fluorescence quenching. The level of fluorescence quenching as a function of MgADP concentration is plotted and shown in Figure 1b. Examination of the plot suggests two successive phases of fluorescence decrease of unequal magnitude (about in 2:1 ratio), followed by a plateau representing 50% of the initial fluorescence. This baseline fluorescence is likely due to the remaining 7 tryptophan and 80 tyrosine residues.

The first phase of fluorescence decrease can be considered as resulting of two overlapping phases, corresponding to filling the first 2 catalytic sites and the last phase corresponded to filling the third catalytic site. The data were fitted to a model involving the successive filling of three sites of decreasing affinity for MgADP, each site contributing to 16.6% of the total fluorescence. The best fit gave K_d values for the three catalytic sites as: K_d₁=0.010±0.002 µM, K_d₂=0.22±0.03 µM, K_d₃=16.3±0.2 µM. The mathematical model used, (see Appendix) takes into account that at the lowest MgADP concentrations used, a significant fraction of nucleotide is bound to the enzyme. The results indicate that catalytic sites of the yeast F₇-ATPase bind MgADP with three unique dissociation constants.

**Occupancy of ATP at the catalytic sites** - While the nucleotide occupancy of the F₇-ATPase with MgADP is important, it is MgATP, not MgADP, which is required for the binding of IF1 to F₇ (31-32). As such, it is important to measure the K_d values for MgATP. However, this is not a trivial measurement since MgATP is rapidly hydrolyzed by the F₇-ATPase. Furthermore at MgATP concentrations from 10-500 µM, steady state ATP hydrolysis is only reached after several minutes (data not shown), probably due to the so-called "hysteric inhibition" (38) followed by the release of inhibitory ADP from catalytic sites (39). These two problems make it difficult to relate the level of the fluorescence quenching to the binding of MgATP or MgADP. Use of a nonhydrolyzable analogue MgAMP-PNP is not the preferred option since steady-state nucleotide occupancy during continuous ATP hydrolysis differs from occupancy of the static enzyme by nonhydrolyzable nucleotide (9).

While quantitative data could not be easily obtained for binding of MgATP, a semiquantitative analysis could be done. In a first step of this analysis, the number of sites that were occupied during ATP hydrolysis at 1 mM MgATP was measured. At this concentration, the ATPase activity is near maximal (see below) and occurs without lag. Kinetics of β-Y345W F₇-ATPase fluorescence quenching with addition of 1 mM MgATP was studied, with focus on the first seconds thereby minimizing the contribution of binding of ADP formed during hydrolysis. To improve the signal/noise ratio, data from 10 experiments were averaged. The results, converted in degree of catalytic sites occupancy, are shown in Figure 2a. Under these conditions, the three catalytic sites are nearly completely (>90%) occupied after 2 seconds with addition of MgATP. Since greater than 90% of the third catalytic sites are rapidly filled with 1 mM MgATP, the apparent K_d of MgATP for the third site must be lower than 100 µM.

A number of control experiments were done to determine if there was significant binding of MgADP under these conditions. The fluorescence quenching with binding of MgADP at 1 mM (Fig. 2b) indicates that MgADP binds somewhat slower to the lowest affinity catalytic site and is complete only after 5-10 s. Thus, if MgADP is present, it binds with a lower rate than that of MgATP. Figure 2c shows the time-course of ADP production by F₇-ATPase at the F₇ and nucleotide concentrations used for the fluorescence experiment in the presence and in the absence of ATP regenerating system. With an ATP regenerating system, the progression
curve is linear while without a regenerating system, ADP production proceeds at the same rate for at least 10 s. The two curves start to diverge between $t=15$ s and $t=20$ s after starting the reaction, due to MgATP decrease and/or to MgADP rebinding. These experiments show that under these conditions, the binding of MgADP does not make a significant contribution to fluorescence quenching of $\beta$-W345.

The affinity of the enzyme for MgATP was also measured by determination of the $K_m$ for ATP hydrolysis. Figure 3 shows a plot of the rate of ATP hydrolysis versus the MgATP concentration. The data fit simple Michaelis-Menten kinetics with a corresponding $K_m$ of 55 $\mu$M and a $V_{\text{max}}$ of 300 s$^{-1}$. The $K_m$ value indicates the concentration of MgATP where 50% of the catalytically active sites are filled. Based on the results of Figures 2 and 3, it is concluded that the $K_m$ represents the $K_d$ of MgATP for the third catalytic site. This indicates that significant ATP hydrolysis only occurs when the third catalytic site is filled with MgATP, i.e. trisite conditions (8-9).

An analysis was done to obtain an estimate of the binding constant for MgATP for the second catalytic site, $K_{d2}$. Again, the intrinsic problem with measuring the binding of MgATP is that it is rapidly hydrolyzed, and at MgATP concentrations from 10-500 $\mu$M, steady state ATP hydrolysis is only reached after several minutes. Thus, the catalytic sites occupancy with MgATP at 1 $\mu$M was estimated and the concentration of $\beta$-Y345W $F_1$-ATPase was lowered to 5 nM to reduce the rate of ATP hydrolysis. Under these conditions, only 13% of the ATP was calculated as hydrolyzed in 5 s and 24% of the ATP in 10 s. Figure 4a shows kinetics of fluorescence quenching with the filling of the catalytic sites after addition of 1 $\mu$M MgATP. About 1.5 sites per enzyme was rapidly occupied and then the occupancy of the catalytic site slowly increased to reach a final value of about 1.8. The kinetics of the fluorescence quenching due to binding MgAMP-PNP (Fig. 4c) shows that MgAMP-PNP binds rapidly to one site and slower to the second site. However, after steady state conditions are achieved (Fig 4d), binding of MgAMP-PNP and MgADP in this concentration range is comparable and the $K_{d2}$ values are nearly identical, that is 0.22 $\mu$M for MgADP.

To summarize, the $K_{d2}$ for MgATP is about 1 $\mu$M and the $K_{d3}$ is about 55 $\mu$M while the $K_{d1}$ for MgADP is 0.01 $\mu$M, the $K_{d2}$ is 0.22 $\mu$M, and the $K_{d3}$ is 16 $\mu$M. The $K_{d2}$ value for MgAMP-PNP is about the same as it is for MgADP, 0.22 $\mu$M. Because of the intrinsic rate of ATP hydrolysis, it has not been possible to estimate the $K_{d1}$ for MgATP binding to the yeast $F_1$. However, it is known to be in the nM range for the E. coli enzyme, or even lower for the mitochondrial enzyme (Ref. 40 for review).

**Binding of IF1 to nucleotide-free $F_1$-ATPase or during single site ATP hydrolysis** - With the binding constants in hand, experiments were undertaken to determine the relationship between nucleotide occupancy of the catalytic site and the binding of IF1. IF1 does not significantly bind to the mitochondrial $F_1$-ATPase in the absence of MgATP (31). This may be due to the lack of accessibility of the binding site on $F_1$ to IF1 or to the quick release of bound IF1. These possibilities were tested by the following experiment. IF1 was bound to the $F_1$-ATPase by incubation of IF1 with $F_1$ in the absence of MgATP. The reaction mixture was then highly diluted in buffer containing MgATP. The dilution minimizes the rebinding of IF1 and the MgATP traps IF1 that is bound to $F_1$. The amount of IF1:$F_1$ complex is then determined based on the initial rate of ATP hydrolysis by $F_1$ free of IF1. Thus, IF1 ($3 \mu$M) was incubated with nucleotide-free $\beta$-Y345W $F_1$-ATPase (40 nM). After indicated incubation times (25°C, pH 6.5), samples were withdrawn and diluted by 125-fold in reaction medium containing 5 mM MgATP and ATP-regenerating system. The rate of ATP hydrolysis was measured immediately to minimize the rebinding of free IF1 to $F_1$. Typical recordings are shown in Figure 5a. The initial
rate of ATP hydrolysis is plotted as a function of incubation time of β-Y345W F₁-ATPase with IF1 (Fig. 5b). There is some inhibition of ATPase activity indicating that IF1 binds to F₁-ATPase devoid of nucleotides. However, the rate of binding is very slow, taking 20 min or more. The curve can be fitted to a first order decay curve and gives a rate constant of 0.001 s⁻¹. By contrast, in the presence of 1 mM MgATP under similar conditions, all of the F₁-ATPase is bound with IF1 in less than a few seconds (not shown).

Using the same approach, we have checked IF1 binding to F₁-ATPase catalyzing single-site ATP hydrolysis. β-Y345W F₁-ATPase (40 nM) was incubated in a medium containing MgATP (50 nM), 5 mM PEP and 200 U/mL pyruvate kinase. IF1 (3 µM) was added, and aliquots were taken up at different times to be checked as previously. IF1 binds to F₁ under conditions where a single catalytic site is filled about 3 fold faster (kₚₕ is 0.0035 s⁻¹) than in the nucleotide-free enzyme (Fig. 5b), yet much slower than at high MgATP concentration (see below). Assuming that kₚₕ is proportional to IF1 concentration (37, 41), the rate constant of IF1 binding to F₁-ATPase, kₜₙ, can be expressed as the ratio between kₚₕ and IF1 concentration. Under nucleotide free conditions, kₜₙ is 0.33 x 10³ s⁻¹M⁻¹ while it is 1.2 x 10³ s⁻¹M⁻¹ under unisite conditions.

Relationship between the MgATP concentration under bisite and trisite conditions and rate of inhibition by IF1 - Figure 6 shows the relationship between MgATP concentrations and the rate of IF1 inhibition. Figure 6a shows typical kinetics of ATP hydrolysis by β-Y345W F₁-ATPase, at three different MgATP concentrations, before and after IF1 (75 nM) addition. The activities before IF1 addition were used to build the plot of Figure 3. The decay of activity after IF1 addition again obeys first-order kinetics, with an apparent rate constant of inhibition kₚₕ (37). In Figure 6b, the apparent second order rate constant, kₜₙ, has been plotted as a function of MgATP concentration. The kₜₙ under bi- and trisite conditions is greater by more than 2 orders of magnitude as that under unisite conditions, going from 0.33 x 10³ s⁻¹M⁻¹ under nucleotide free conditions and from 1 x 10⁵ s⁻¹M⁻¹ under bisite conditions (1 µM MgATP) to a maximum of 4 x 10⁵ s⁻¹M⁻¹ under trisite (20 µM MgATP) conditions. Using the established binding constants, at 1 µM MgATP 50% of the molecules have 1 MgATP bound to the catalytic site and at 20 µM, 70% have 2 and 30% have 3 molecules of MgATP at the catalytic sites. These results indicate that IF1 binds very rapidly to F₁ bound under bi- and trisite conditions as compared to unisite conditions.

At MgATP concentrations increasing beyond about 50 µM, the rate of inhibition by IF1 decreases. The decrease in IF1 inhibition rate with increasing ATP concentrations is paradoxical but this general curve has also been observed for the reaction of bovine IF1 with bovine F₁ (32). At least part of this inhibition may be attributed to the transition from bisite conditions to trisite conditions. Figure 6b shows the relationship between the filling of the second and third catalytic sites and change in kₚₕ for binding of IF1 to F₁. The kₚₕ increases with the filling of the second catalytic site and then starts to fall when 50% of the molecules have 2 catalytic sites filled and 50% have 3 catalytic sites filled. The fall in kₚₕ continues even beyond 1 mM MgATP where nearly 100% of molecules have the third catalytic site filled. Thus, there must be something else contributing to the decrease in the binding of IF1 to F₁ at the higher MgATP concentrations. The decrease in kₜₙ at higher MgATP concentrations might be related to the change in the ionic strength of the reaction medium (42).

The effect of the ionic strength on the binding of IF1 with F₁ was determined by the rate of IF1 inhibition with increasing the MgCl₂ concentrations in the presence of background 40 µM MgATP. From MgCl₂ additions of 20 µM to 200 µM, there was negligible effect on kₜₙ. However, the kₜₙ decreased by about 40% at 2 mM MgCl₂ and about 70% at 5 mM MgCl₂ and above (data not shown). Thus, the increasing ionic strength due to the increase in concentration of MgATP may be the cause for the decrease in the kₜₙ at the higher MgATP concentrations.

Overall, the results indicate that IF1 binds slowly to F₁ that is devoid of nucleotide or with just one catalytic site filled with nucleotide. The rate of binding to F₁ is accelerated by 2
orders of magnitude in the transition from unisite to bisite conditions and then slightly decreases when the majority of the molecules have 3 catalytic sites filled. The binding of IF1 to F1 is also dependent on the ionic strength of the medium and decreases steadily beyond 1 mM salt.

DISCUSSION

The goal of these studies is to provide a better understanding of the mechanism of inhibition of the natural inhibitor peptide, IF1, with the yeast F1-ATPase. IF1 is a rather unique inhibitor because while it is able to inhibit the hydrolysis of ATP by F1 or F0F1, it does not inhibit the synthesis of ATP by F0F1 (43, 2). In addition, IF1 is critical in the mammalian cells and its expression is regulated in ischemic hearts (44-45). Thus, there is considerable interest in IF1 for mechanistic and practical reasons. The studies presented here were designed to correlate the nucleotide content of the F1-ATPase with the rate of binding and inhibition of IF1 thereby giving an insight in the dynamics of the inhibition mechanism.

Nucleotide occupancy in the catalytic sites of the F1-ATPase - The occupancy of the catalytic sites was measured by the fluorescence quenching of a Trp residue, which was engineered in the catalytic site. This method was used many times by Senior's group for the E. coli F1-ATPase (9, 46-51). Quenching of this residue upon the binding of nucleotide was sufficient to measure binding of nucleotides, even though there are seven other Trp residues in the molecule. The binding constants of MgADP were the first measurements made using this method. This determination was relatively easy measurement since the fluorescent quenching upon binding nucleotide was large and MgADP is not hydrolyzed upon binding to the active site. The results provided the first accurate measurement of the binding affinity of a mitochondrial F1. This is important because the nucleotide binding properties of the E. coli enzyme significantly differ from those of the mitochondrial enzyme as seen in this and other studies (9, 46, 52). The results indicate that the mitochondrial enzyme has three unique nucleotide binding constants of $K_{d1}=0.01 \mu M$, $K_{d2}=0.22 \mu M$, $K_{d3}=16.3 \mu M$. The $K_{d2}$ value determined here is similar to that obtained by equilibrium binding methods (53). These values indeed differ significantly from those obtained for the E. coli enzyme for which the corresponding $K_d$ values for MgADP were 0.05 µM and 29 µM with one nucleotide binding to the tight site and 1.4 nucleotides binding to the weaker sites (9). Thus, the mitochondrial enzyme binds MgADP much tighter as compared to the E. coli enzyme and shows much stronger cooperativity, especially in the binding of the third nucleotide (9). A partial analysis was also done on the binding of MgAMP-PNP. This analysis indicated that MgAMP-PNP bound to yeast F1 with a $K_{d2}$ similar to that of MgADP. This too is consistent with the results that were obtained with the E. coli enzyme (9).

Although it is relatively easy to measure the binding of MgADP or MgAMP-PNP, it is much more difficult to measure the binding of MgATP to the yeast F1-ATPase. ATP is rapidly hydrolyzed by F1 at concentrations required for fluorescence detection making the binding of MgADP a confounding variable. This was not such a problem in the study using the E. coli enzyme because of its relatively low rate of ATP hydrolysis, but the yeast mitochondrial enzyme hydrolyzes ATP 15 fold faster than the E. coli enzyme (300 sec$^{-1}$ vs 20 sec$^{-1}$) (54). Another problem is that there are time-dependent changes in the active site of the mitochondrial enzyme, which are likely due to ADP inhibition and subsequent activation (39). Thus, we were limited in the analysis on the binding of MgATP. Despite these restrictions, we were able to get estimates of the $K_d$ values for MgATP. Based on the binding of MgATP at 1 mM and 1 µM and combined with the measured $K_m$ value, it was concluded that $K_{d2}$ and $K_{d3}$ were 1 µM and 55 µM. For comparison, the $K_{d2}$ value determined for the E. coli enzyme is 0.5 µM (8). The $K_m$ value is also nearly identical to that obtained earlier for the yeast enzyme (55) and $K_{d3}$ value for the E. coli enzyme (9). We were unable to obtain $K_{d1}$ from this study because of the very high affinity of this site for MgATP. However, prior studies indicate that the yeast enzyme has nearly identical nucleotide binding properties under unisite conditions as the bovine mitochondrial enzyme (53) and the $K_{d1}$ for
MgATP for the bovine enzyme is $10^{-12}$ M (52). For the current analysis, we used a $K_d$ value of 10 nM, which is the value determined here for MgADP, though a lower $K_d$ value for MgATP, which is highly probable, would not change the conclusions made here.

Another significant conclusion based on the binding and hydrolysis data of MgATP is that the mitochondrial F$_1$-ATPase is active for ATP hydrolysis only when all three sites are filled. This provides yet additional support for the trisite model (8-9) of ATP hydrolysis as opposed to the bisite model (7, 10-11).

**Mechanism of IF1 binding to F$_1$** - The rate constant ($k_{on}$) for IF1 binding to the F$_1$-ATPase or F$_0$F$_1$ ATP synthase can provide insight into the accessibility of the IF1 binding site and how the binding site changes during catalysis or with nucleotide binding. Analysis of the $k_{on}$ has been useful for the analysis for other enzymes undergoing large conformation changes including the analysis of the interaction between thioredoxin and chloroplast ATP synthase (56-57). Turnover-dependent variations of $k_{on}$ may suggest which catalytic intermediates bind IF1, as first suggested by the work of Milgrom (32) (see also ref. (58)). This rationale formed the basis of this study to relate $k_{on}$ for the binding of IF1 to F$_1$ with the nucleotide occupancy of the F$_1$-ATPase.

The results from these experiments provide some new and important information on the mechanism of IF1 binding to F$_1$. First, IF1 binds to nucleotide depleted F$_1$ or to F$_1$ under unisite conditions (Fig. 5), but extremely slowly as compared to multisite conditions. This suggests that under multisite catalysis, IF1 does not bind to a site on F$_1$ that is devoid of nucleotide, $\beta_{DE}$. The results also suggest that IF1 does not bind well to the nucleotide binding site that has the highest affinity for ATP. Under multisite conditions, IF1 binds quite rapidly, and most effectively when ATP concentrations range from 20-100 $\mu$M MgATP (Fig. 6). At these MgATP conditions, about 50% of the F$_1$ molecules have two catalytic sites occupied and 50% have three catalytic sites occupied. Thus, the binding site of IF1 on the yeast F$_1$-ATPase is dependent on the nucleotide occupancy of the enzyme with rapid binding occurring only if there are 2-3 active sites bound with nucleotide.

**Mechanism of inhibition by IF1** - In order to discuss the binding mechanism of IF1 to F$_1$ under multisite conditions, it is helpful to have a model that encompasses the known details of the enzyme reaction scheme. A minimal model describing multisite catalysis is shown in Figure 7a. This model is based on a number of studies from other laboratories (8-9, 50-51, 59, 30), and is consistent with the studies which indicate that maximal ATP hydrolysis occurs in trisite conditions (51). The state with two catalytic sites occupied with nucleotide, 1, corresponds to the putative ground state as seen in first high-resolution crystal structure of the bovine F$_1$ ATPase (4). This structure has one catalytic site filled with ATP ($\beta_{TP}$), one with ADP ($\beta_{DP}$), and one site is empty ($\beta_{DE}$). The state with three catalytic sites occupied, 2, has two ADPs and one ATP bound. This state is likely predominant during steady state hydrolysis conditions under $V_{max}$ conditions (9, 51). The model also shows the position of the $\gamma$ subunit and includes the results of the single molecule studies that indicate that the binding of ATP to $\beta_{DE}$ causes the $\gamma$ subunit to rotate 80°, and that the final 40° rotation is driven by ATP hydrolysis and/or P$_i$ release (8). The same study also indicates that hydrolysis occurs on $\beta_{TP}$, before release of ADP from $\beta_{DP}$.

The published crystal structure of bovine F$_1$ with IF1 resembles the ground state structure with only two catalytic sites occupied, except that IF1 is bound to $\beta_{DP}$, and $\beta_{TP}$ has bound ATP (or AMP-PNP) instead of ADP (Fig. 7a, 4) (30). Based on this structure, it was proposed that IF1 binds to either $\beta_{DE}$ or $\beta_{EE}$ that has just bound nucleotides and then undergoes two rounds of ATP binding while inhibiting ATP hydrolysis (30). For this to be consistent with the known elements of the catalytic cycle (Fig. 7), the $\gamma$ subunit must rotate 240° in the absence of ATP hydrolysis. This mechanism is thus in conflict with the results of the single molecule experiments, which indicate that the final 40° of the 120° rotation in a catalytic cycle is dependent on ATP hydrolysis or P$_i$ release (8). Inhibition of the step of ATP hydrolysis by IF1 should therefore lead to a dead-end complex as
Alternative hypotheses on the inhibition by IF1 have been proposed and one suggests that IF1 allows the conversion of $\beta_{TP}$ to $\beta_{DP}$ and prevents only the release of ADP (30). This hypothesis is not consistent with crystal structure since ATP (or AMP-PNP) is trapped in the catalytic site with IF1 bound, and not ADP. It might be argued that this mechanism is correct and the ATP bound in the crystal structure was due to the exchange of this nucleotide for ADP during the crystallization procedure (30). However, this mechanism would also predict that the final complex IF1:F1 would contain nucleotides bound to each of the catalytic site and ATP (or AMP-PNP) would be predicted to be bound to $\beta_{E}$, not on $\beta_{DP}$ as seen in the structure (Fig. 7a, state 6). Thus, neither of these hypotheses are consistent with both the crystallographic structure of the bovine F1:IF1 complex (30) and the current view on the catalytic mechanism of ATP hydrolysis (8).

**Catalytic-dependent binding of IF1** - Our results provide new information on the primary events of IF1 binding. In the model based on the crystal structure of bovine F1:IF1 (30), it was proposed that IF1 binds to $\beta_{E}$ before or after MgATP binding to that site. The data of Figure 6 indicate that the binding site of IF1 during trisite ATP hydrolysis is readily accessible in both catalytic states, 1 and 2, with a small decrease in the IF1 binding to 2 with respect to 1. This should be distinguished from the previous proposal that the F1-ATPase with the three catalytic sites filled could be unable to bind IF1 (32). The binding site is not likely present in a transient structure, such as a transition state structure, since IF1 binding is rapid under multisite conditions. In regard to state 1, our results clearly indicate that IF1 cannot be binding to $\beta_{E}$. Thus, either $\beta_{TP}$ or $\beta_{DP}$ may be the IF1 binding site. However, our data from Figure 5 indicates that IF1 does not bind to the F1 with a single nucleotide bound to F1. Since $\beta_{DP}$ is thought to be the site that has the highest affinity for nucleotides, this would suggest that IF1 does not bind to $\beta_{DP}$. There is a caveat because the structure of bovine F1 with just one of the catalytic sites filled with nucleotide has not been determined and $\beta_{DP}$ may not exist in this structure. However, the crystal structure also supports that IF1 binds to $\beta_{TP}$ and not $\beta_{DP}$, because $\beta_{TP}$ is more open and thus accessible to IF1 than is $\beta_{DP}$ (4).

Based on the kinetic data (Fig. 6), IF1 binds to a catalytic site shown in 1 or 2 (Fig. 7). The crystal structure corresponding to 2 has not been determined, so the conformation of the sites that correspond to $\beta_{DP}$ and $\beta_{TP}$ is uncertain. In this structure, the $\gamma$-subunit has rotated just 80° of the 120° rotation. Despite the fact that this is an intermediate in the reaction scheme, this appears to be the major species present during multisite catalysis as we observed that all sites are filled with nucleotide during multisite ATP hydrolysis (Fig. 6) and this is also supported by experiments with the *E. coli* enzyme (9). The most simple model for IF1 binding, based on the results of this study and the prior studies, is that IF1 binds to both structures shown in 1 and 2, and that IF1 binds to $\beta_{TP}$, or what was most recently $\beta_{TP}$, but this still remains speculative. If IF1 does bind to $\beta_{TP}$, then there is still a requirement that $\beta_{TP}$ is converted to $\beta_{DP}$ to obtain the structure of the bovine F1:IF1 complex as represented in Figure 7a, 4, but this will only require 120° rotation by the $\gamma$-subunit and not a 240° rotation as in the prior mechanism.

**Distinction between loose binding and locking of IF1** - Our data (Fig. 6) also strongly suggest a two-step mechanism in the formation of the IF1-F1 complex during ATP hydrolysis (Fig. 7b). In the first step, IF1 binds F1 at what we believe is $\beta_{TP}$. At this point, IF1 is loosely bound and can release from the binding site. In the second step, there is a turnover-dependent locking of the binding site giving a tight IF1-F1 complex. The rate of IF1 binding to F1 that we observe results from the binding rate constant ($k_{on}$ in Fig. 7b) modulated by the competition between release ($k_{off}$) and locking ($k_{lock}$), the locking rate increasing with turnover rate (see Equation of Fig. 7b and Appendix). If the turnover is low, there is no inhibition by IF1, even with two catalytic sites filled with nucleotides. On the other hand, the maximal rate of inhibition by IF1
is obtained before saturation of the three catalytic sites, provided the rate of IF1 release ($k_{off}$) becomes negligible with respect to the rate of locking ($k_{lock}$). This scheme explains why the $k_{on}$ increases with increasing concentration of MgATP without being strictly correlated to the filling of the second or the third catalytic site with nucleotide (Fig. 6).

This two-step mechanism also provides a simple explanation for the 1:1 stoichiometry of IF1 bound to F$_1$ (60-61). If the locking step is much faster than the binding IF1 to F$_1$, then once a molecule of IF1 is bound and locked in place, then hydrolysis is inhibited thereby preventing the locking of a second IF1 to F$_1$. This explanation is valid regardless the catalytic interface that primarily binds IF1 and is based on the fact that newly bound IF1 cannot be locked into an already inactivated F$_1$-ATPase complex.

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APPENDIX

1 - Modelling the relationship between the nucleotide concentration and fluorescence of β-Y345W F1-ATPase.

F1-ATPase is assumed to exist under four different states called E, ES1, ES1S2 and ES1S2S3, linked by successive equilibria with the dissociation constants $K_{d1}<K_{d2}<K_{d3}$. The only difficulty comes from the fact that total and free nucleotides cannot be considered as equal at low concentrations (10-100 nM). At higher concentrations, this simplification can be made. Nucleotide-free enzyme concentration is the positive root of the following equation:

$$
(1 + 2 \frac{[S_{tot}]}{K_{d2}})[E]^2 + \left[ [S_{tot}](1 + \frac{[S_{tot}]}{K_{d2}}) + K_{d1} - [E_{tot}](1 + 2 \frac{[S_{tot}]}{K_{d2}}) \right] [E] - K_{d1}[E_{tot}] = 0 \quad (Eqn \, 2)
$$
where \( S_{\text{tot}} \) is the total nucleotides, \( E_{\text{tot}} \) is the total enzyme, and \( E \) the nucleotide-free enzyme. One gets:

\[
[E] = \frac{-b + \sqrt{b^2 - 4ac}}{2a}
\]

(Eqn 3)

with \( a = 1 + 2 \frac{[S_{\text{tot}}]}{K_d^2} \),

(Eqn 4)

\[ b = [S_{\text{tot}}](1 + 2 \frac{[S_{\text{tot}}]}{K_d^2}) + K_d [E_{\text{tot}}] - [E_{\text{tot}}](1 + 2 \frac{[S_{\text{tot}}]}{K_d^2}) \]

(Eqn 5)

\[ c = -K_d [E_{\text{tot}}] \]

(Eqn 6)

The sum of concentrations of all the nucleotide-loaded states [ES123], is simply given by:

\[ [ES123] = [E_{\text{tot}}] - [E] \]

(Eqn 7)

To calculate [ES1], [ES2] and [ES3] from [ES1S2S3], the \( K_{d1} \) constant is no more necessary. So the free and total nucleotide concentrations can now be considered as the same. One obtains:

\[ [ES1S2S3] = \frac{[ES123]}{[S_{\text{tot}}] + 1 + \frac{[S_{\text{tot}}]}{K_d^3}} \]

(Eqn 8)

\[ [ES1] = [ES1S2S3] \frac{K_d^2}{[S_{\text{tot}}]} \]

(Eqn 9)

\[ [ES1S2S3] = [ES1S2S3] \frac{[S_{\text{tot}}]}{K_d^3} \]

(Eqn 10)

The fluorescence \( F \) of \( \beta \)-Y345W F1-ATPase is given by:

\[ F = F_0 - \Delta F \frac{3[E_{\text{tot}}] + 2[ES1S2] + 3[ES1S2S3]}{[E_{\text{tot}}]} \]

(Eqn 11)

where \( F_0 \) is the fluorescence of \( \beta \)-Y345W F1-ATPase in the absence of nucleotides, and \( \Delta F \) is the fluorescence quenched by the filling of a single catalytic site.

2 - Modelling the two-step binding of IF1.

The kinetic scheme is the following:

\[
E + I \overset{k_{\text{on}}}{\underset{k_{\text{off}}}{\rightleftharpoons}} EI \overset{k_{\text{lock}}}{\rightarrow} EI'
\]

Where \( E \) is the active enzyme and \( I \) the inhibitory peptide. In the first step, \( I \) loosely binds to \( E \) to form \( EI \). In the second step, \( I \) is irreversibly locked in the enzyme, which gives the form \( EI' \). The variations of \([E] \) and \([EI']\) are given by the following equations:

\[
\frac{d[E]}{dt} + k_{\text{on}}[E][I] = k_{\text{off}}[EI]
\]

(Eqn. 12)

\[
\frac{d[ EI' ]}{dt} = k_{\text{lock}}[EI]
\]

(Eqn. 13)

Eqns. 12-13 give:

\[
\frac{d[E]}{dt} + k_{\text{on}}[E][I] = \frac{k_{\text{off}}}{k_{\text{lock}}} \frac{d[ EI' ]}{dt}
\]

(Eqn. 14)

We assume \( k_{\text{on}}[E][I] \ll k_{\text{off}} \). Then \([EI]\) can be neglected at any time, and one can write:

\[
\frac{d[E]}{dt} = - \frac{d[ EI' ]}{dt}
\]

(Eqn. 15)

Equation 14 becomes:

\[
\frac{d[E]}{dt} \left(1 + \frac{k_{\text{off}}}{k_{\text{lock}}} \right) + k_{\text{on}}[E][I] = 0
\]

(Eqn. 16)
The solution of this equation is:

\[ [E] = [E_0] e^{-k_{on}t} \]  

(Eqn. 17)

with

\[ \frac{k_{on}}{1 + \frac{k_{off}}{k_{lock}}} \]  

(Eqn. 18)

\( k_{on} \) is the experimentally measured binding rate constant. \( k_{lock} \) increases with enzyme turnover (then with MgATP concentration). \( k_{on} \) becomes equal to \( k_{on} \) when \( k_{lock} > k_{off} \).

**FOOTNOTES**

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1The abbreviations used are: F₀F₁, ATP synthase complex (EC 3.6.3.14); F₀, membranous sector of ATP synthase; F₁, catalytic sector of ATP synthase; AMP-PNP, adenylyl-imidodiphosphate; Pᵢ, inorganic phosphate; EDTA, ethylenediaminetetraacetic acid; MES, 2-(N-morpholino)ethane sulfonic acid; Tris, tris (hydroxymethyl) aminomethane.

**FIGURE LEGENDS**

**Fig. 1.** Nucleotide occupancy probed by fluorescence decrease of tryptophan β-345. a: time course of fluorescence decrease upon nucleotide addition to F₁-ATPase. Nucleotide additions are indicated by vertical arrows. Curve 1, wild-type F₁-ATPase; curves 2-5, β-Y345W F₁-ATPase. The conditions used are as described in "Experimental Procedures". λₑₓc=295 nm; λₑₘ=350 nm. b: Fluorescence of β-Y345W F₁-ATPase as a function of MgADP concentration. F₁ concentration was 20 nM (for MgADP 0.02-3 µM) or 30 nM (for MgADP 3-10,000 µM). The data was corrected for the internal filter effect of nucleotides and expressed as percentage of the fluorescence of nucleotide-free β-Y345W F₁-ATPase. The continuous curve represents the fitting of the data based on a 3-sites binding model. The resulting parameters are: \( K_{d1}=10±2 \) nM, \( K_{d2}=0.22±0.03 \) µM, \( K_{d3}=16.3±0.2 \) µM, fluorescence quenching per site: 16.6±0.4 %. See "Results" and "Appendix" for more details.

**Fig. 2.** Time-course of catalytic sites occupancy and ATP hydrolysis at 1 mM MgATP. a and b: time-courses of catalytic sites occupancy upon addition of 1 mM MgATP (a) and 1 mM MgADP (b) to 30 nM β-Y345W F₁-ATPase. a: 10 kinetics averaged; b: 4 kinetics averaged. One datapoint per second in both cases. Nucleotide additions are indicated by vertical arrows. Nucleotide occupancy was calculated from fluorescence decrease on the basis of the calibration curve of Figure 1b. The conditions as described under "Experimental Procedures" and Fig. 1. c: ATP hydrolysis by β-Y345W F₁-ATPase in the presence (■) and in the absence (□) of ATP regenerating system.

**Fig. 3.** Steady state rate of ATP hydrolysis as a function of MgATP concentration. β-Y345W F₁-ATPase used at a concentration of 1.8 nM (for MgATP ranging from 2 µM to 20 mM), 3.6 nM (for MgATP 1 µM) and 7.2 nM (for MgATP 0.5 µM). ATPase activity, expressed in number of turnovers per second, was fitted to a Michaelis-Menten model with \( V_{max}=300 \) s⁻¹ and \( K_m=55 \) µM. Conditions are as described in "Experimental Procedures" and "Results".
Fig. 4. Time-course of catalytic sites occupancy after adding 1 µM MgATP or 1 µM MgADP, and MgAMP-PNP in the micromolar range. a: time-course of catalytic sites occupancy upon addition of 1 µM MgATP to 5 nM β-Y345W F1-ATPase, 10 kinetics averaged. Conditions are as described in "Experimental Procedures" and Fig. 2. b: time-course of catalytic sites occupancy upon addition of 1 µM MgADP, 10 kinetics averaged. One datapoint per second in both cases. Nucleotide occupancy was calculated from fluorescence decrease using the same principle as in Fig. 3. c: time-course of catalytic sites occupancy upon addition of 1 µM MgATP or 1 µM MgADP (■), or MgAMP-PNP (−Δ−, data and fit replotted from Fig. 2b). F1-ATPase concentration was 5 nM in a-b and 30 nM in c-d.

Fig. 5. Binding of IF1 to F1-ATPase at low catalytic site occupancy revealed at high MgATP concentration. a: time-course of ATP hydrolysis upon dilution of pretreated nucleotide-free F1-ATPase into the reaction medium containing 5 mM MgATP. Vertical arrow, F1-ATPase addition in the spectrophotometric cuvette. Curve 1, control (no IF1); curves 2-4, F1-ATPase preincubated with 3 µM IF1 for 1 min (2), 7 min (3) and 20 min (4); black trace 1 and grey traces 2-3-4, spectrophotometric recordings; black curves 2-3-4, fits used to calculate the initial rates. Conditions are as described in "Experimental Procedures". b: initial rates of ATP hydrolysis, calculated as shown in (a), as a function of the time of preincubation with 3 µM IF1; (○), preincubation without MgATP; (●), preincubation in the presence of 50 nM MgATP. Values normalized to those obtained after the same time of incubation in the absence of IF1.

Fig. 6. Rate constant of inhibition by IF1 as a function of MgATP concentration and filling of the catalytic sites. a: time course of ATP hydrolysis and ATPase inhibition monitored by NADH absorbance at 340 nm; curves 1, 2 and 3 correspond to MgATP 1 µM, 40 µM and 2 mM, respectively. Steady state rates of ATP hydrolysis were used to build the plot of Fig. 3. Time of IF1 addition (75 nM) is indicated by vertical arrows. A small absorbance decrease upon IF1 addition, due to dilution, is visible on curve 1. Kinetic analysis used to calculate the apparent rate constant inhibition has been corrected from this absorbance drop. Conditions are as described in "Experimental Procedures" and "Results". β–Y345W F1-ATPase used at a concentration of 1.8 nM (for MgATP ranging from 2 µM to 20 mM), 3.6 nM (for MgATP 1 µM) and 7.2 nM (for MgATP 0.5 µM). b: apparent rate constant of IF1 binding (■) as a function of MgATP concentration. Apparent binding rate constant, equivalent to $k_{on}$, was obtained by dividing the apparent rate constant of inhibition ($k_{app}$) by IF1 concentration (75 nM). The two dashed curves represent the proportion of the F1-ATPases with two (left curve) and three (right curves) bound nucleotides, assuming $K_{d1}$=10 nM, $K_{d2}$=0.2 µM and $K_{d3}$=55 µM. See text for more details.

Fig. 7. ATP hydrolysis and IF1 binding. a: The two main catalytic intermediates during trisite ATP hydrolysis seen from the membrane side. The nomenclature of the sites ($\beta_{DP}$, $\beta_{TP}$, $\beta_{E}$) is that of Ref (4), except that $\beta_{DP}$ also contains P. State 1, State 2, intermediates with two or three catalytic sites occupied, respectively. In State 2 the central axis has rotated counterclockwise by 80° with respect to State 1. State 3 is equivalent to State 1 after a 120° rotation of the central axis. The accessibility of the IF1 binding site is thought to be somewhat lower in State 2 than in State 1. State 4 is the dead-end state inhibited by IF1 as deduced from the crystallographic structure of F1-ATPase in complex with IF1 (30). State 5 is the expected structure of the dead-end state inhibited by IF1 if IF1 blocks ATP hydrolysis on the catalytic site where it has been bound and subsequent 40° rotation of the central axis (8). The position of the central axis is the same as in State 2. State 6 is the expected structure of the dead-end state inhibited by IF1 if IF1 allows ATP hydrolysis on the catalytic site where it has been bound and prevents ADP release from the vicinal site, provided replacement of ADP by AMP-PNP on the new $\beta_{DP}$ site later occurs during crystallization (30) (AMP-PNP has been replaced here by its equivalent ATP for sake of simplicity). Neither State 5, nor State 6 are superimposable to the structure of State 4. b: kinetic model of IF1 binding to F1-ATPase. The inhibitory peptide (black rectangle) slowly binds ($k_{on}$) to the enzyme. Once bound, IF1
can be either released ($k_{\text{off}}$) or locked ($k_{\text{lock}}$) within the enzyme. The latter process is turnover-dependent and almost irreversible at acidic pH. With two catalytic sites filled, $k_{\text{on}}$ is somewhat higher than the highest value plotted on Figure 1 b ($k_{\text{on}} \approx 5 \times 10^5 \text{ s}^{-1} \text{ M}^{-1}$). With three catalytic sites filled, it may be somewhat lower. At low IF1 concentration (below $\mu$M), IF1 binding occurs in tens of seconds or minutes. Release and locking are much faster, probably well below seconds range. In the absence of enzyme turnover, loosely bound IF1 does not stay on the enzyme and no binding can be observed. When the rate of turnover (then $k_{\text{lock}}$) becomes high enough, quasi-irreversible binding is observed. The experimentally observed binding rate constant ($k'_{\text{on}}$) is given by the formula: $k'_{\text{on}} = \frac{k_{\text{on}}}{1 + \frac{k_{\text{off}}}{k_{\text{lock}}}}$ and therefore tends to $k_{\text{on}}$ at

high enzyme turnover (see Appendix).
FIGURE 1
FIGURE 2

(a) The effect of MgATP on the number of occupied sites.

(b) The effect of MgADP on the number of occupied sites.

(c) The reaction time (s) versus ATP hydrolyzed (µM).

FIGURE 2
FIGURE 3
FIGURE 4

(a) MgATP

(b) MgADP

(c) MgAMP-PNP

(d) Number of occupied sites vs. [MgATP] (µM)
FIGURE 6
FIGURE 7
The binding mechanism of the yeast F1-ATPase inhibitory peptide. Role of catalytic intermediates and enzyme turnover

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