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Conformational Changes of the Mitochondrial F_1-ATPase ϵ-Subunit Induced by Nucleotide Binding as Observed by Phosphorescence Spectroscopy*

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Changes in conformation of the ϵ-subunit of the bovine heart mitochondrial F_1-ATPase complex as a result of nucleotide binding have been demonstrated from the phosphorescence emission of tryptophan. The triplet state lifetime shows that whereas nucleoside triphosphate binding to the enzyme in the presence of Mg^{2+} increases the flexibility of the protein structure surrounding the chromophore, nucleoside diphosphate acts in an opposite manner, enhancing the rigidity of this region of the macromolecule. Such changes in dynamic structure of the ϵ-subunit are evident at high ligand concentration added to both the nucleotide-depleted F_1 (Nd-F_1) and the F_1 preparation containing the three tightly bound nucleotides (F_1(2,1)). Since the effects observed are similar in both the F_1 forms, the binding to the low affinity sites must be responsible for the conformational changes induced in the ϵ-subunit. This is partially supported by the observation that the Trp lifetime is not significantly affected by adding an equimolar concentration of adenine nucleotide to Nd-F_1. The effects on protein structure of nucleotide binding to either catalytic or noncatalytic sites have been distinguished by studying the phosphorescence emission of the F_1 complex prepared with the three noncatalytic sites filled and the three catalytic sites vacant (F_1(3,0)). Phosphorescence lifetime measurements on this F_1 form demonstrate that the binding of Mg-NTP to catalytic sites induces a slight enhancement of the rigidity of the ϵ-subunit. This implies that the binding to the vacant noncatalytic site of F_1(2,1) must exert the opposite and larger effect of enhancing the flexibility of the protein structure observed in both Nd-F_1 and F_1(2,1). The observation that enhanced flexibility of the protein occurs upon addition of adenine nucleotides to F_1(2,1) in the absence of Mg^{2+} provides direct support for this suggestion. The connection between changes in structure and the possible functional role of the ϵ-subunit is discussed.

The ATPase (ATP synthase) is the enzyme responsible for ATP synthesis during oxidative phosphorylation in all energy-transducing membranes. It is composed of two main parts: F_0, capable of proton transport across the membrane and the catalytic part; F_1, bound to F_0 through a "stalk" segment (for reviews see Refs. 1–5). F_1 of eukaryotes and prokaryotes are similar in subunit composition; they are composed of five different subunits α through ϵ, in order of decreasing molecular weight, with the stoichiometry 3, 3, 1, 1, and 1 (2, 6). However, these enzymes are not identical mainly because of differences in two of the subunits; δ- and ϵ-subunits of bacteria (and chloroplasts) are homologous to oligomycin sensitivity conferring protein and δ of beef heart, respectively. Therefore, the ϵ-subunit of mitochondria lacks a counterpart in bacteria and chloroplasts. The two major subunits α and β of F_1 contain at their interfaces a total of six nucleotide binding sites, which are characterized by different properties (7–13). Nucleotide sites have been classified as catalytic or noncatalytic according to their ability to exchange bound ligand rapidly during hydrolysis of Mg-ATP (13). According to this definition, there are three catalytic and three noncatalytic sites. The functional role of the latter sites has yet to be elucidated. However, several authors have suggested a regulatory function (11, 14, 16–18). Only two noncatalytic sites and one catalytic site are fully occupied on desalted F_1; these sites are regarded as tight binding sites (19), and this state of occupancy is described by the symbol F_1(2,1)[1] according to Kironde and Cross (20). The vacant noncatalytic site is also regarded as the exchangeable noncatalytic site (21). Catalytic sites have been shown to exhibit magnesium dependence and a relatively broad nucleotide specificity (2, 19, 22), whereas noncatalytic sites have a significant preference for adenine nucleotides (19, 20, 23–25).

Both ATP hydrolysis and ATP synthesis catalyzed by the F-type ATPases are cooperative processes now thought to involve ligand-induced and energy-dependent conformational changes, which modulate the affinity of catalytic sites for substrates and products (25, 26). The mechanism and the particular domain (or subunit) of F_1 and F_0 involved in each individual step of the processes are unknown, in particular the mode of the transmission of conformational signals between domains of the protein. Approaches that have been used to monitor conformational changes in F_1 and in F_0F_1-ATPase preparations, including chemical labeling of certain amino acid residues of the protein (27, 28), hydrogen exchange (29), binding of inhibitors (30), and protease digestion experiments (31, 32), are invasive, thus providing information regarding a structurally altered protein. Methods are needed to selectively monitor conformational changes of specific domains or subunits of the unmodified protein.

Trp phosphorescence measured at room temperature has shown considerable potential for the study of protein structure in solution (33). The sensitivity of the triplet-state lifetime of

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[1] The abbreviations used are: $F_1(x,y)$, F_1 containing x mol of ANP at noncatalytic sites and y mol of ANP at catalytic sites per mol of enzyme; F_1, soluble part of the F-type H^+-ATPases; Nd-F_1, nucleotide-depleted F_1; AMP-PNP, 5′-adenylyl-β,γ-imidodiphosphate; HPLC, high pressure liquid chromatography.

the indole nucleus to the flexibility of its surrounding matrix (34) has been extremely useful with respect to revealing subtle conformational changes induced in proteins by binding of substrates, coenzymes, inhibitors, or interacting macromolecules (35–37). We have recently used the phosphorescence of the sole tryptophan residue of the mitochondrial F_1 complex as an internal probe of the ϵ-subunit (38, 39), and the lifetime (τ) measurements have revealed conformational changes of the nucleotide-depleted enzyme as a consequence of Mg-ATP binding at low temperature.

The high complexity of nucleotide binding sites of F_1 and the existence of temperature-dependent conformational states of the enzyme prompted us to further investigate on possible alterations in the dynamic structure of the ϵ-subunit induced by selective nucleotide-site occupancy. We have analyzed the phosphorescence decay kinetics of F_1 at room temperature in the presence or absence of nucleoside di- and triphosphates associated with loose or tight, catalytic and noncatalytic nucleotide binding sites. These noninvasive studies of intrinsic phosphorescence provide evidence that the conformation of the ϵ-subunit *in situ* is affected differently by the binding of nucleoside di- or triphosphates to the various nucleotide binding sites of F_1.

MATERIALS AND METHODS

ATP, ADP, GTP, GDP, phosphoenolpyruvate, Hepes, Tris, and NADH were obtained from Sigma as were pyruvate kinase and lactate dehydrogenase in glycerol-containing buffers. Sephadex G-50, Sephacryl S-300, Blue Sepharose CL-6B, and standard marker proteins were obtained from Pharmacia Biotech Inc.

F_1 was prepared from bovine heart mitochondria according to Penefsky (40). We have observed that at this stage the enzyme preparation contained minor contaminants: a protein with an apparent molecular mass of about 48 kDa and the ATPase inhibitor protein. All of the contaminants were removed as follows. By affinity chromatography on Blue Sepharose CL-6B, 16 mg of protein was loaded onto a column (4 cm × 1 cm, inner diameter) in 0.2 M NaCl, 1 mM EDTA, 1 mM β-mercaptoethanol, 1 mM ATP, and 20 mM Tris-Cl, pH 8 (41), at a 4 ml/h flow rate. 13 mg of the non-retained protein were concentrated to 15 mg/ml by ultrafiltration with a Diaflo XM-300 (Amicon) membrane. The concentrate was then chromatographed on Sephacryl S-300 (40 cm × 1.6 cm, inner diameter) in 25 mM Tris-Cl, 0.25 M sucrose, and 1 mM ATP, pH 8; a typical elution profile is shown in Fig. 1*A*. Fractions containing the 32–40-ml elution volume (9 mg of protein) were characterized by a constant specific activity, indicating the presence of a single molecular species that was used in the experiments. SDS-polyacrylamide gel electrophoresis confirmed this assertion since even overloading the gel, the typical five-subunit pattern of F_1 was observed (Fig. 1*B*). The enzyme solution was stored at 5 °C as a suspension at 50% ammonium sulfate saturation in the presence of 4 mM ATP (pH 8). The enzyme activity was stable for several weeks in this state. Since the technique used in our experiments evaluates the single Trp of F_1, it is extremely important that no contaminating protein is present in the enzyme preparation to avoid Trp signals other than that of F_1.

F_1(2,1) was obtained from the above enzyme suspension by centrifugation, and the sedimented enzyme was dissolved at 4–6 μM in a buffer containing 150 mM sucrose, 1 mM KH_2PO_4, 1 mM $MgSO_4$, 10 mM K^+-Hepes, pH 8; it was desalted on a Sephadex G-50 centrifuge column (42) equilibrated with the same buffer. F_1(3,0) was prepared from the ammonium sulfate suspension removing unbound nucleotide and desalting by passage through a centrifuge column equilibrated with 150 mM sucrose, 10 mM Hepes, 1 mM $MgSO_4$, pH 8; it was then followed by a procedure based on the displacement of nucleotides from the catalytic sites by a brief exposure (1 min) to pyrophosphate and removal of unbound nucleotides by gel filtration (20). F_1(3,0) was also prepared from F_1(2,1) by substituting pyrophosphate with Mg-GDP, according to Ref. 43. Nd-F_1 was prepared from submitochondrial particles by gel permeation chromatography in the presence of 50% glycerol (v/v) as previously described (38). All of the F_1 preparations were pure and had the typical subunit stoichiometry as evidenced from SDS-polyacrylamide gel electrophoresis (Fig. 1*B*). This is of particular importance, since it has been hypothesized that the ϵ-subunit might be substoichiometric with respect to the rest of the protein (12), which could result in misinterpretation of the phosphorescence data. The percent volume calculated for the protein bands of a typical F_1, using the Molecular Analyst PC image analysis software for the Bio-Rad densitometer (model GS-670) was $\alpha + \beta = 85.81$, $\gamma = 9.49$, $\delta = 3.41$, and $\epsilon = 1.29$.

The nucleotide content of each enzyme preparation was determined by reverse-phase HPLC, following nucleotide extraction according to Di Pietro *et al.* (44). The HPLC analysis of the extracts was performed on a Nova-Pak™ C_{18} column (150 × 3.9 mm, 4-μm Nova-Pak™ packing material, Waters) equilibrated in 30 mM KH_2PO_4, pH 5.4, containing 3 mM tetrabuthylammonium sulfate. Nucleotides were eluted with a 0–30% acetonitrile gradient over 25 min at a flow rate of 1 ml/min. Detection was by absorbance at 260 nm. The nucleotide/F_1 molar ratios observed were 0.4 ± 0.1 for Nd-F_1, 2.8 ± 0.4 for F_1(2,1), and 2.6 ± 0.5 for F_1(3,0) using a molecular mass of 370 kDa- for the enzyme (6) in all stoichiometry calculations.

SDS-polyacrylamide gel electrophoresis was carried out according to Laemmli (45) using a polyacrylamide gradient from 14 to 25% containing 0.1% SDS. The procedure has been previously described in detail (46).

The ATPase activity was determined with an ATP regenerating system by following the decrease of NADH absorption at 340 nm in a 7850 model Jasco spectrophotometer. The assay was carried out at substrate-saturating concentration (steady state) as previously reported (38). The specific activity of the enzyme was 80–100 units/mg protein at 20 °C.

Protein concentrations of enzyme solutions were determined by the method of Lowry *et al.* (47).

Phosphorescence spectra and decay measurements were obtained with a phosphorimeter, constructed in this institution, as previously described (48). The photons were generated by a Cernax xenon lamp (LX 150UV, ILC Technology), and the wavelength was set with a 250-nm grating monochromator (Jobin-Yvon, H25). The emission was detected with an EMI 9635 QB photomultiplier. Phosphorescence decay in fluid solution at room temperature was monitored with a homemade apparatus suitable for lifetime measurements in the μs–ms range described in detail elsewhere (75). Pulsed excitation(λ_{ex} = 292 nm) was generated by a frequency-doubled flash-pumped dye laser (UV500 M Candela) with a pulse duration of 1 μs and an energy/pulse typically of 1–10 mJ. The sample, placed in a vacuum-proof quartz cuvette that allows excitation of the solution from above, is extensively deoxygenated prior to analysis. The emitted light is measured at 90° from the excitation light and selected by a filter combination in the window between 420 and 480 nm. The photomultipliers are protected from the intense excitation light and fluorescence pulse by a high speed chopper blade that closes the slits during laser excitation. The minimum lag time of the apparatus is about 10 μs. The decay signal was digitized by a computerscope system (ISC-16, RC Electronics) capable of averaging multiple sweeps. Subsequent analysis of decay curves in terms of the sum of exponential components was carried out by a nonlinear least squares fitting algorithm implemented by the program Global Analysis (Global Unlimited, LFD University of Illinois, Urbana).

For each sample, the phosphorescence decay was measured three times, and samples were prepared at least four times. The standard error of preexponential terms and lifetime components are better than ± 10%. It should be noted, however, that the variability of these parameters can be even somewhat greater when one compares different preparations of the protein. Such variability in the decay kinetics can be traced down to different amounts of quenching impurities present in organic solvents (glycerol), and glasswares (75). For this reason, comparisons are always made between samples obtained from the same enzyme preparation.

RESULTS

Nucleotide Effects on Nd-F_1 Phosphorescence Decay—Fig. 2 shows the selected examples of phosphorescence intensity decay of Nd-F_1 incubated with adenine nucleotides 1 mM at 293 K. The lifetimes (τ) and the preexponential parameters (α) derived from a biexponential curve fitting are shown in Table I. Incubation of Nd-F_1 with adenine nucleotides at 1 mM in the presence of magnesium changes the intrinsic phosphorescence decay parameters of Nd-F_1. ATP decreases the average phosphorescence lifetime from 2.8 to 1.9 ms. This lifetime decrease indicates an enhanced flexibility of the polypeptide chain surrounding the chromophore (35, 49, 50). Thus, the addition of ATP to Nd-F_1 induces conformational changes of the protein, resulting in a more flexible environment for the N-terminal segment of the ϵ-subunit, where Trp is located at position 4 (6).

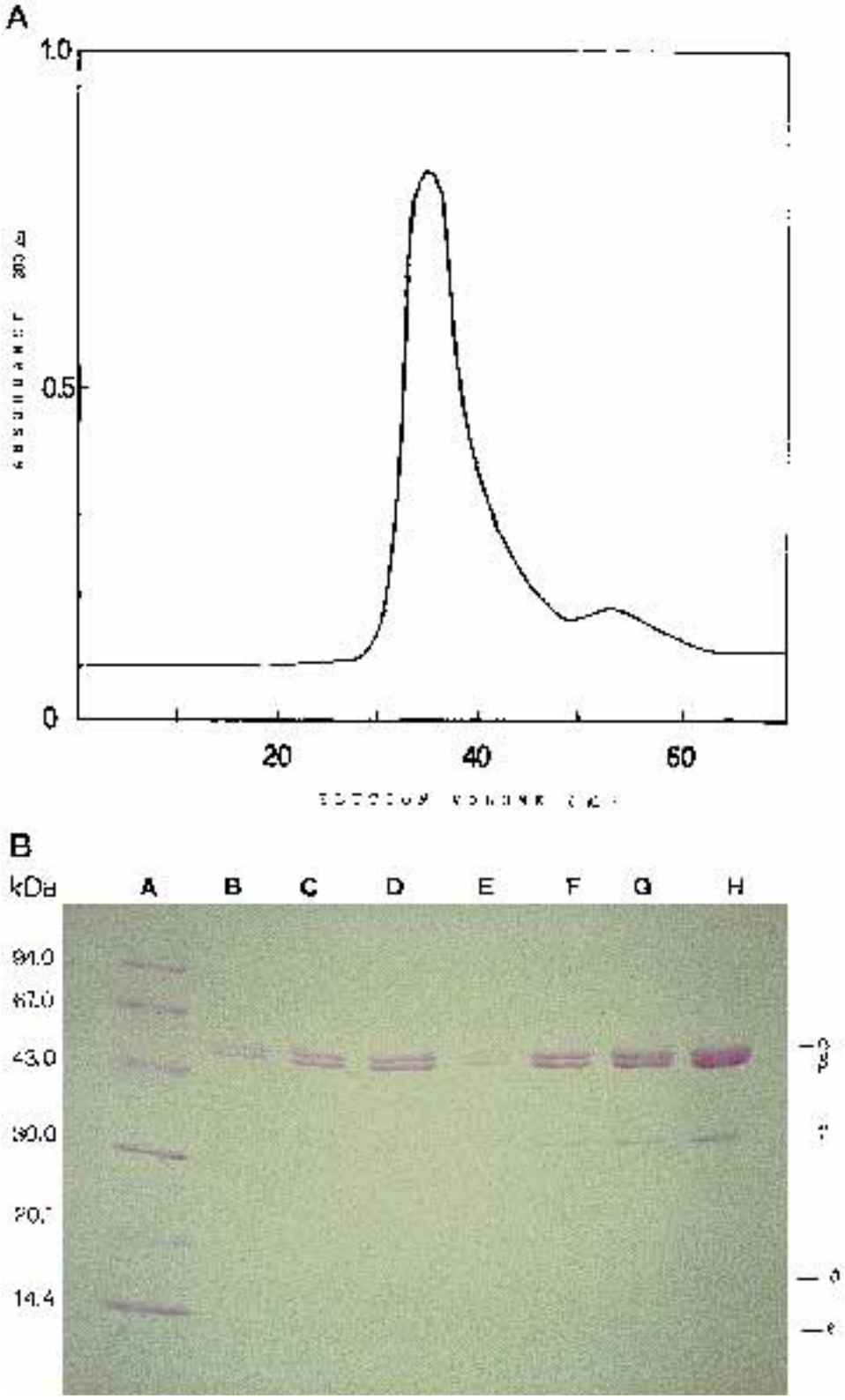

FIG. 1. **Purity of the F_1 complex.** *A*, elution profile of the complex passed through a Sephacryl S-300 column (see "Materials and Methods"). *B*, the samples were analyzed by SDS-polyacrylamide gel electrophoresis (45) and stained with Coomassie Brilliant Blue R-250. *Lanes A*, standard marker proteins (Pharmacia) are as follows: rabbit muscle phosphorylase *b* (94.0 kDa), bovine serum albumin (67.0 kDa), egg white ovalbumin (43.0 kDa), bovine erythrocyte carbonic anhydrase (30.0 kDa), soybean trypsin inhibitor (20.1 kDa), bovine milk α-lactalbumin (14.4 kDa); *lanes B–D*, 4, 6, and 10 μg of Nd-F_1, respectively; *lanes E* and *F*, 3 and 9 μg of F_1(3,0), respectively; lanes *G* and *H*, 15 and 20 μg of F_1(2,1), respectively. The positions of the F_1 subunits are indicated at the *right* side.

ADP has an effect opposite that of ATP; it enhances the protein rigidity since the intrinsic τ_{av} of the protein increases from 2.8 of Nd-F_1 to 3.4 ms upon nucleotide binding.

The opposite effect induced on the ϵ-subunit conformation by ATP and ADP binding suggests that different conformations of the nucleotide binding sites are induced by the two nucleotides and that different allosteric effects are then transmitted to the Trp environment of the ϵ-subunit. This would be consistent

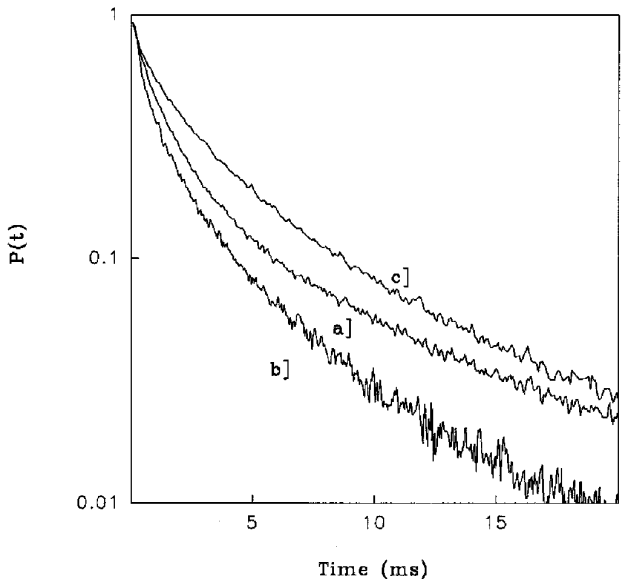

FIG. 2. **Decay of phosphorescence intensity of Nd-F_1 in the presence of Mg-ANP at 293 K.** In *a* is shown Nd-F_1, in *b*, 1 mM Mg-ATP is added to Nd-F_1, and in *c*, 1 mM Mg-ADP is added to Nd-F_1. Experimental details are reported in the legend to Table I.

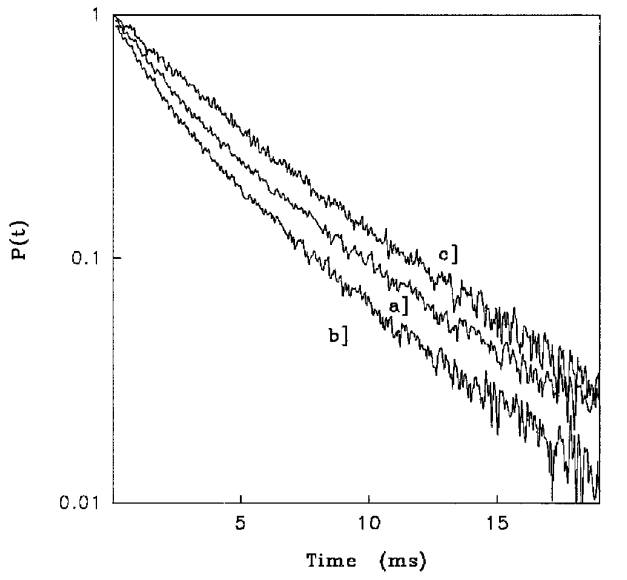

FIG. 3. **Decay of phosphorescence intensity of F_1(2,1) in the presence of Mg-ANP at 293 K.** *a*, F_1(2,1); *b*, F_1(2,1) in the presence of 1 mM Mg-ATP; and *c*, F_1(2,1) in the presence of 1 mM Mg-ADP. Experimental details are reported in the legend to Table I.

TABLE I

Magnesium nucleotide effects on F_1-ATPase phosphorescence decay

NdF_1 (4 μM) was dissolved in 100 mM Tris/SO_4^-, 0.5 mM EDTA, and 10% glycerol, pH 8. F_1 (2,1) (4 μM) was in 150 mM sucrose, 1 mM $MgSO_4$, and 10 mM K^+-Hepes, pH 8. F_1 (3,0) (3 μM) was in 150 mM sucrose, 1 mM $MgSO_4$, 10 mM Hepes, pH 8. Triplet state lifetimes (τ_i) and preexponential terms (α_i) are derived from a biexponential fitting of the phosphorescence decay ($P(t) = \alpha_1 e^{-t/\tau_1} + \alpha_2 e^{-t/\tau_2}$) of F_1 form added with Mg^{2+} (2 mM) and with nucleotide. τ_{av} is the average phosphorescence lifetime ($\tau_{av} = \tau_1\alpha_1 + \tau_2\alpha_2$). Phosphorescence decays (λ_{ex} = 292 nm) were measured at 20 °C.

F_1 form	Ligand	Concentration	Phosphorescence decay				
			τ_1	α_1	τ_2	α_2	τ_{av}
		mM	ms		ms		ms
NdF_1			1.4	0.84	10	0.16	2.8
NdF_1	Mg-ATP	1	1.1	0.85	6.2	0.15	1.9
NdF_1	Mg-GTP	$5{\cdot}10^{-1}$	1.2	0.86	7.4	0.14	2.1
NdF_1	Mg-ADP	1	1.9	0.78	8.6	0.22	3.4
NdF_1	Mg-ADP	$3.7{\cdot}10^{-3}$	1.3	0.75	6.1	0.25	2.5
NdF_1	Mg-ATP	$3.7{\cdot}10^{-3}$	1.3	0.85	11	0.15	2.7
F_1 (2,1)			2.7	0.71	8.2	0.29	4.3
F_1 (2,1)	Mg-ATP	1	1.5	0.44	4.8	0.56	3.3
F_1 (2,1)	Mg-GTP	$5{\cdot}10^{-1}$	1.6	0.54	5.7	0.46	3.5
F_1 (2,1)	Mg-ADP	1	3.3	0.67	7.4	0.33	4.6
F_1 (2,1)	Mg-ADP + P_i	1	3.2	0.58	7.0	0.42	4.8
F_1 (3,0)			1.0	0.8	5.9	0.2	2.0
F_1 (3,0)	Mg-ATP	1	1.3	0.72	5.8	0.28	2.5
F_1 (3,0)	Mg-GTP	$5{\cdot}10^{-1}$	0.8	0.7	5.9	0.3	2.3

with the idea of several authors who, on the basis of inhibition studies (18, 51–52), speculated that the F_1-ATPase complex may exist into two different conformations, E_s and E_h, favored by ADP and ATP binding, respectively.

When Nd-F_1 is incubated with stoichiometric amounts of ADP in the presence of Mg^{2+}, it results in inhibition. It has been shown that the inhibitory ADP is bound in a catalytic site (52–54). To establish whether the binding of ADP to this high affinity catalytic site affects the conformation of the ϵ-subunit, ADP has been added stoichiometrically to Nd-F_1. The decay parameters (Table I) do not change significantly with respect to control, indicating a lack of influence of ADP filling the high affinity catalytic site on the conformation of the protein at the ϵ-subunit level. Also, the addition of stoichiometric ATP to Nd-F_1 (*i.e.* conditions for unisite catalysis as in Ref. 55) does not affect the Trp phosphorescence decay. Therefore, independent of the adenine nucleotide tested, it would appear reasonable to conclude that filling the high affinity catalytic site does not result in conformational changes of the protein involving the N-terminal segment of the ϵ-subunit.

To further investigate which nucleotide site(s) of F_1 have to be occupied to induce ϵ-subunit conformational changes, preparations of F_1 at different levels of nucleotide occupancy of sites have been studied.

F_1(2,1) Phosphorescence Decay Changes Induced by Nucleotides—Preliminarily, we obtained the phosphorescence spectra of the F_1(2,1) form in glycerol-phosphate buffer glass at 140 K (not shown), and we observed that the spectra are essentially identical to that of Nd-F_1 previously reported (38). This implies that the nucleotide binding to the high affinity sites does not change the physico-chemical environment of the ϵ-subunit Trp.

Incubation of F_1(2,1) with 1 mM Mg-ADP, 1 mM Mg-ATP, or 0.5 mM Mg-GTP at 293 K in 150 mM sucrose, 1 mM $MgSO_4$, 10 mM Hepes, pH 8, induces consistent changes of the phosphorescence decay (Fig. 3). Table I shows the phosphorescence decay parameters of a typical experiment. The data clearly display a significant decrease of the average lifetime from 4.3 to 3.3 and 3.5 ms when ATP or GTP are bound, respectively. This indicates an increased flexibility of the chromophore environment upon occupancy of the vacant nucleotide binding sites by NTP, whereas ADP has an opposite effect since it enhances τ_{av} to 4.6 ms (Table I). Thus, the results are similar to those observed on Nd-F_1, providing further support to the conclusion that 1) occupation of loose binding site(s) is responsible for the ϵ-subunit structural change and 2) binding of the nucleoside triphosphate increases the flexibility of the Trp environment, whereas the binding of nucleoside diphosphate enhances its rigidity (Mg^{2+} present).

It has been shown that addition of ATP or ADP plus P_i in the presence of magnesium results in a reactivation of the AMP-PNP-inhibited ATP hydrolysis activity of the enzyme (56). The similar behavior of ATP or ADP plus P_i has prompted investigation with respect to similar effect of the ligands on the F_1 conformation. Thus, experiments designed to determine whether inorganic phosphate added together with ADP might have the capability to influence the ϵ-subunit conformation

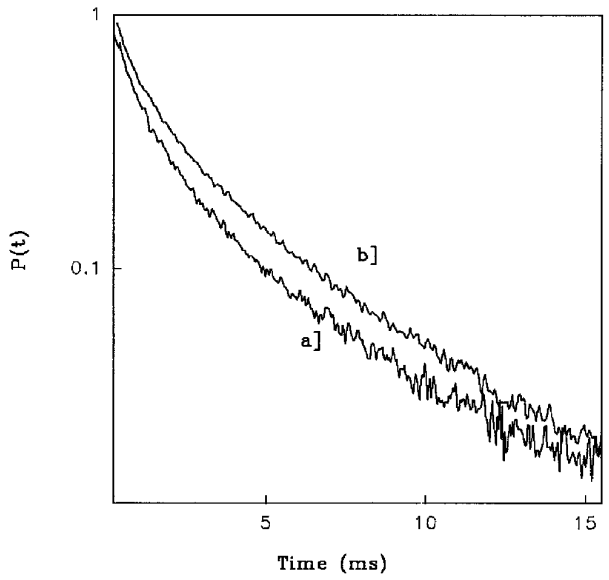

FIG. 4. **Decay of phosphorescence intensity of F_1(3,0) in the presence of Mg-ATP at 293 K.** *a*, F_1(3,0); *b*, F_1(3,0) in the presence of 1 mM Mg-ATP. Experimental details are reported in the legend to Table I.

similarly to ATP were initiated. For this purpose, F_1 was incubated in the presence of ADP + Mg^{2+} over a range of 0.1–10 mM P_i (*i.e.* the physiological range, according to Refs. 57 and 58), with care being taken to exclude adventitious P_i from enzyme and buffers. This treatment did not alter significantly the effect of ADP alone on the phosphorescence decay parameters of the enzyme (Table I), indicating that the activation effect of P_i on the Mg-ADP-F_1 complex cannot be related to the ϵ-subunit conformation.

F_1(3,0) Phosphorescence Decay Changes Induced by Nucleotide Binding—To establish whether the effects observed are the consequence of nucleotide binding to either or both the catalytic and the noncatalytic sites left vacant in the F_1(2,1) form, F_1(3,0), the enzyme containing three filled noncatalytic sites and three vacant catalytic sites, was prepared following two different procedures using either pyrophosphate or GDP in the presence of Mg^{2+} to displace selectively nucleotides from the catalytic sites. The results obtained using one or the other of the two preparations were similar. Fig. 4 shows a typical decay curve obtained incubating at 293 K the enzyme in the presence of Mg-ATP. The addition of both ATP and GTP in the presence of Mg^{2+} results in increased τ_{av}, which shifts from 2 ms for F_1(3,0) to 2.5 and 2.3 ms, respectively (Table I). Therefore, filling catalytic sites with nucleoside triphosphates induces a significant tightening of the ϵ-subunit conformation.

The comparison of these data with those obtained on Nd-F_1 suggests that the effects observed on addition of nucleotides to both Nd-F_1 and F_1(2,1) are the sum of two distinct effects; the binding of the exchangeable noncatalytic site induces an increased flexibility of the ϵ-subunit N-terminal domain, which likely overwhelms the tightening of the same domain induced on filling the loose catalytic binding sites.

F_1(2,1) Phosphorescence Decay in the Absence of Mg^{2+} under Different Nucleotide Conditions—Noncatalytic nucleotide binding sites of F_1 have a preference for uncomplexed nucleotides (*i.e.* in the absence of magnesium ions) (21, 59–61). Here, we describe the effects of incubating the enzyme with adenine nucleotides, in the absence of Mg^{2+}, on the ϵ-subunit conformation. Control experiments show that the phosphorescence emission decay of F_1(2,1) with excess EDTA has a similar biexponential behavior as in the presence of excess Mg^{2+}. This indirectly indicates that the binding of Mg^{2+} to F_1(2,1) does not affect the Trp environment of the ϵ-subunit. Moreover, the phosphorescence decay parameters of F_1(2,1), to which was added EDTA + P_i, do not show significant changes of the ϵ-subunit conformation (Table II).

TABLE II
Effects of nucleotide binding on F_1 (2,1) phosphorescence decay in the absence of Mg^{2+} ions

F_1 (2,1) (4 μM) was in 150 mM sucrose and 10 mM K^+-Hepes, pH 8. EDTA (2 mM) was added to all samples. Nucleotide concentration was 1 mM. Potassium phosphate effect on the enzyme lifetime was checked in the range 0.1–10 mM. Experimental conditions and parameters are detailed in the legend to Table I.

Ligand	Phosphorescence decay				
	τ_1	α_1	τ_2	α_2	τ_{av}
	ms		*ms*		*ms*
	2.7	0.71	8.2	0.29	4.3
P_i	2.1	0.34	5.1	0.66	4.1
ATP	2.2	0.72	7.6	0.28	3.7
ADP	1.8	0.67	4.8	0.33	2.8
ADP + P_i	1.5	0.49	3.7	0.51	2.6

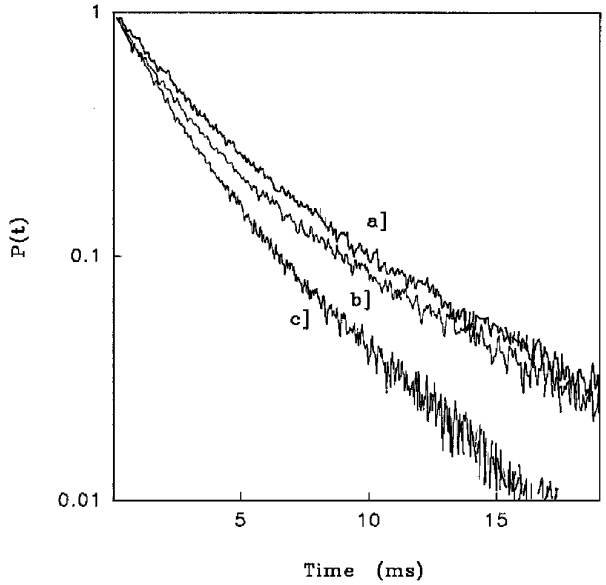

FIG. 5. **Decay of phosphorescence intensity of F_1(2,1) in the presence of ANP (Mg^{2+} absent) at 293 K.** *a*, F_1(2,1) was in 150 mM sucrose, 10 mM K^+-Hepes, 2 mM EDTA, pH 8; *b*, 1 mM was added to F_1(2,1); and *c*,1 mM ADP was added to F_1(2,1).

Addition of ATP in the presence of 2 mM EDTA reduces the τ_{av} value from 4.3 to 3.7 (Fig. 5 and Table II). This result was expected on the basis of above results where the filling of the exchangeable noncatalytic nucleotide binding sites could reduce τ_{av}, indicating an enhanced flexibility of the protein. The enhanced flexibility associated with the filling of the exchangeable noncatalytic site and the enhanced rigidity associated to the filling of loose catalytic sites might be additive or not. In fact, it seems they are not since if they were, one would have expected an even larger increase of the protein flexibility, for the filling of the catalytic sites under the present experimental conditions is not favorable. This divergence from additivity might be explained if one considers that Mg^{2+} changes the structure of the nucleotide binding sites as recently shown (62) and that the binding of sites in the absence of Mg^{2+} might result in structural changes transmitted to the ϵ-subunit different from those observed in the presence of the metal.

Similar considerations could explain the effect of ADP on the dynamic properties of F_1 in the absence of magnesium ions, as monitored by Trp phosphorescence. In fact, addition of the nucleoside diphosphate in the absence of Mg^{2+} reduces τ_{av} from

4.3 to 2.8 ms (Table II), indicating an increased flexibility of the protein, whereas an enhancement of rigidity was observed in experiments in which ADP was added along with Mg^{2+} to the F_1-ATPase complex.

Finally, addition of 0.1–10 mM P_i along with (or following) ADP does not change significantly the effect of ADP when added alone. Therefore, as in the presence of Mg^{2+}, in its absence the addition of inorganic phosphate to the ADP-F_1 complex is without effect on the ϵ-subunit conformation.

DISCUSSION

The ϵ-subunit of the mitochondrial F_1-ATPase complex is the polypeptide with the lowest molecular mass (5.5 kDa) of the protein, and its function is unknown. However, it is thought that it plays a role in the coupling between F_1 and F_0 (12, 63). Consistent with such a role, although experimental evidence of the ϵ-subunit location is not available, most of the F_1 and F_0F_1 models show the polypeptide located at the interior of the α-β-subunit core, facing F_0, possibly contributing to the stalk region (3, 5, 12, 51, 64, 65).

Previously, through the investigation of the intrinsic phosphorescence of the mitochondrial F_1-ATPase complex, we showed that the addition of Mg-ATP to the enzyme bearing vacant nucleotide binding sites resulted in large conformational changes of the protein surrounding the N-terminal domain of the ϵ-subunit (38, 39). However, our experiments were performed at 273 K or below in the presence of 50% glycerol as a stabilizing co-solvent to operate under optimal conditions for the measurement of the phosphorescence signal. Given these conditions, it is not possible to interpret our results in terms of *in vivo* function, since the kinetic properties and the conformation of F_1 in media characterized by high viscosity and below 16 °C are quite different from those shown between 20 and 37 °C (28, 66).

In the present study, we have overcome the problem since we were able to carry out the experiments at 20 °C by modifying equipment for phosphorescence measurements, and we extended the investigations analyzing the effect of filling with different nucleotides several enzyme forms characterized by different nucleotide content and configuration.

This research has revealed a few intriguing features concerning conformational changes of the ϵ-subunit upon binding of nucleotides to the mitochondrial F_1 complex. 1) The binding of nucleotides to the loose sites is solely responsible for the conformational changes observed on the ϵ-subunit. This contention is based on the following observations: first, addition of Mg-ATP or Mg-GTP in large molar excess to both the Nd-F_1 and the enzyme containing the tightly bound nucleotides, F_1(2,1), produces a net shortening of the phosphorescence lifetime, comparable in the two enzyme preparations; second, incubation of Nd-F_1 with unistoichiometric Mg-ATP, which loads a single, high affinity catalytic site, does not affect the average phosphorescence lifetime of F_1; third, phosphorescence spectra of F_1(2,1) are identical to those of Nd-F_1. 2) The comparative analysis of the results obtained with F_1(2,1), F_1(3,0), and Nd-F_1 (this in the presence of unistoichiometric Mg-ATP) strongly suggests that the increased flexibility of the Nd-F_1 and F_1(2,1) forms upon binding of Mg-ATP or Mg-GTP is in fact the result of two different effects and that the filling of the vacant noncatalytic site is responsible for the large increased flexibility of the ϵ-subunit. These conclusions are supported by results of the experiment carried out in the absence of magnesium, a condition favoring the binding of nucleotides to the noncatalytic sites. Under this condition, the average phosphorescence lifetime of F_1(2,1) markedly decreases upon incubation with adenine nucleotides. 3) Mg-ADP addition to the ATPase complex consistently and greatly enhances the rigidity of the Trp microenvironment, showing an opposite effect with respect to Mg-ATP. Thus, our results provide evidence for two opposite conformational changes of the mitochondrial F_1-ATPase ϵ-subunit whether ATP + Mg^{2+} or ADP + Mg^{2+} is added. Moreover, addition of P_i along with (or following) ADP + Mg^{2+} did not significantly alter the effect of ADP + Mg^{2+} only, suggesting that once Mg-ADP is bound, P_i can not influence the ADP-F_1 structure as probed at the ϵ-subunit level.

The markedly different conformational changes obtained on binding of Mg-ATP, as compared to Mg-ADP, might have several possible explanations. Thus, F_1 might assume two conformational states, depending whether ADP or ATP is the ligand. This possibility has been proposed by Boyer (51) on the basis of various experimental observations reporting significant differences in the ATPase behavior, whether the enzyme has been examined in the presence of ADP or ATP. Interestingly, an x-ray crystallographic study of a Ras protein catalyzing GTP hydrolysis has shown substantial structural differences whether Mg-GTP or Mg-GDP was the ligand. These differences seemed to be caused by a different coordination of the active site Mg^{2+} ion (67). Since F_1 shares with the Ras protein the conserved phosphate-binding loop (68), Mg^{2+} might have a similar role with F_1 on binding Mg-ATP or Mg-ADP. A second possibility is that different types of metal-nucleotide diastereoisomers could be the true ligands for binding Mg-ADP or Mg-ATP to F_1 (69). It has indeed been shown that different metal-nucleotide epimers of ADP and ATP are the substrates for a number of F_1-ATPases (70). The consequence might be that different structural signals could be transmitted from the nucleotide binding sites of F_1 to the ϵ-subunit, depending on the particular type of stereoisomer bound.

If the ϵ-subunit is not in close proximity of the nucleotide binding sites, as it is believed, our results demonstrate that conformational changes of F_1 upon substrate binding are transmitted over long distances. Thus, the ϵ-subunit might be involved in the propagation of signals to deeper regions of the F_0F_1 complex.

Finally, observations of certain similarities between the present study and those carried out on F_1 from other sources should be cited. Evidence for a correlation between occupation of the nucleotide binding sites, catalysis, and conformational changes of the ϵ-subunit were obtained in studies with both *Escherichia coli* and chloroplast F_1 (31, 32, 72, 73).

The enzyme from the different sources has the functional core $\alpha_3\beta_3\gamma$ composed of homologous subunits and has two additional small, single-copy subunits, which appear to play a role in the energy-coupling mechanism and are physically close to one another (15, 64, 71, 74). The mitochondrial F_1 ϵ-subunit, which has no counterpart in other species, might share with the ϵ-subunit of F_1 from other sources the involvement in the coupling mechanism and/or in its regulation. Nevertheless, the molecular events involving conformational changes of the ϵ-subunit of F-type ATPases from different energy-transducing membranes might differ from one another, as it can be envisaged if one considers several different effects observed on small subunits of mitochondria and *E. coli* F_1 upon filling of the nucleotide sites. Mendel-Hartvig and Capaldi (31) found a direct relationship between P_i binding and ϵ-subunit conformation of *E. coli* F_1, whereas it was not possible to find any such relationship with the mitochondrial enzyme. Moreover, both Mg-ADP and Mg-ATP could induce a less tight structure of the ϵ-subunit of *E. coli*, whereas in our study Mg-ADP and Mg-ATP could increase or decrease, respectively, the tightness of the mitochondrial protein.

In conclusion, our results provide information on dynamic aspects of the enzyme structure and function and provide the

first noninvasive, extensive experimental evidence for a change in the conformation of the mitochondrial F_1-ATPase ϵ-subunit *in situ* in response to the filling of different classes of nucleotide binding sites with several substrates.

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REFERENCES

1. Boyer, P. D. (1993) *Biochim. Biophys. Acta* **1140,** 215–250
2. Senior, A. E. (1988) *Physiol. Rev.* **68,** 177–231
3. Penefsky, H. S., and Cross, R. L. (1991) *Adv. Enzymol.* **64,** 173–214
4. Hatefi, Y. (1993) *Eur. J. Biochem.* **218,** 759–767
5. Issartel, J. P., Dupuis, A., Garin, J., Lunardi, J., Michel, L., and Vignais, P. V. (1992) *Experientia* **48,** 351–362
6. Walker, J. E., Fearnley, I. M., Gay, N. J., Gibson, B. W., Northrop, F. D., Powell, S. J., Runswick, M. J., Saraste, M., and Tybulewicz, V. L. J. (1985) *J. Mol. Biol.* **184,** 677–701
7. Harris, D. A. (1978) *Biochim. Biophys. Acta* **463,** 245–273
8. Wise, J. G., Duncan, T. M., Latchney, L. R., Cox, D. N., and Senior, A. E. (1983) *Biochem. J.* **215,** 343–350
9. Issartel, J. P., Lunardi, J., and Vignais, P. V. (1986) *J. Biol. Chem.* **261,** 895–901
10. Bullough, D. A., Brown, E. L., Saario, J. D., and Allison, W. S. (1988) *J. Biol. Chem.* **263,** 14053–14060
11. Di Pietro, A., Penin, F., Godinot, C., and Gautheron, D. C. (1980) *Biochemistry* **19,** 5671–5678
12. Abrahams, J. P., Leslie, A. G. W., Lutter, R., and Walker, J. E. (1994) *Nature* **370,** 621–628
13. Cross, R. L., and Nalin, C. M. (1982) *J. Biol. Chem.* **257,** 2874–2881
14. Xue, Z., and Boyer, P. D. (1989) *Eur. J. Biochem.* **179,** 677–681
15. Richter, M. L., Snyder, B., McCarty, R. E., and Hammes, G. G. (1985) *Biochemistry* **24,** 5755–5763
16. Milgrom, Y. M., Ehler, L. L., and Boyer, P. D. (1990) *J. Biol. Chem.* **265,** 18725–18728
17. Harris, D. A. (1993) *FEBS Lett.* **316,** 209–215
18. Jault, J-M., and Allison, W. S. (1994) *J. Biol. Chem.* **269,** 319–325
19. Harris, D. A., Gomez-Fernandez, J. C., Klungsoyr, L., and Radda, G. K. (1978) *Biochim. Biophys. Acta* **504,** 364–383
20. Kironde, F. A. S., and Cross, R. L. (1986) *J. Biol. Chem.* **261,** 12544–12549
21. Kironde, F. A. S., and Cross, R. L. (1987) *J. Biol. Chem.* **262,** 3488–3495
22. Rosing, J., Harris, D. A., Slater, E. C., and Kemp, A. (1975) *Biochim. Biophys. Acta* **376,** 13–26
23. Perlin, D. S., Latchney, L. R., Wise, J. G., and Senior, A. E. (1984) *Biochemistry* **23,** 4998–5003
24. Milgrom, Y. M., and Cross, R. L. (1993) *J. Biol. Chem.* **268,** 23179–23185
25. Rosen, G., Gresser, M., Vinkler, C., and Boyer, P. D. (1979) *J. Biol. Chem.* **254,** 10654–10661
26. Cross, R. L., Grubmeyer, C., and Penefsky, H. S. (1982) *J. Biol. Chem.* **257,** 12101–12105
27. Stan-Lotter, H., and Bragg, P. D. (1986) *Eur. J. Biochem.* **154,** 321–327
28. Baracca, A., Solaini, G., Dinelli, G., Parenti-Castelli, G., and Lenaz, G. (1993) *Int. J. Biochem.* **25,** 701–706
29. Ryrie, I. J., and Jagendorf, A. T. (1972) *J. Biol. Chem.* **247,** 4453–4459
30. Issartel, J. P., Klein, G., Satre, M., and Vignais, P. V. (1983) *Biochemistry* **22,** 3485–3490
31. Mendel-Hartvig, J., and Capaldi, R. A. (1991) *Biochemistry* **30,** 1278–1284
32. Bragg, P. D., and Hou, C. (1987) *Biochim. Biophys. Acta* **894,** 127–137
33. Strambini, G. B. (1989) *J. Mol. Liq.* **42,** 155–165
34. Strambini, G. B., and Gonnelli, M. (1985) *Chem. Phys. Lett.* **115,** 196–200
35. Cioni, P., and Strambini, G. B. (1989) *J. Mol. Biol.* **207,** 237–247
36. Strambini, G. B., and Gabellieri, E. (1987) *Biochemistry* **26,** 6527–6530
37. Gabellieri, E., and Strambini, G. B. (1994) *Eur. J. Biochem.* **221,** 77–85
38. Solaini, G., Baracca, A., Parenti-Castelli, G., and Strambini, G. B. (1993) *Eur. J. Biochem.* **214,** 729–734
39. Baracca, A., Barogi, S., Gabellieri, E., Lenaz, G., and Solaini, G. (1995) *Biochem. Biophys. Res. Commun.* **207,** 369–374
40. Penefsky, H. S. (1975) *Methods Enzymol.* **55,** 304–308
41. Lutter, R., Abrahams, J. P., Van Raaij, M. J., Todd, R. J., Lundqvist, T., Buchanan, S. K., Leslie, A. G. W., and Walker, J. E. (1993) *J. Mol. Biol.* **229,** 787–790
42. Penefsky, H. S. (1977) *J. Biol. Chem.* **252,** 2891–2899
43. Peinnequin, A., Issartel, J. P., Lunardi, J., and Vignais, P. V. (1992) *Biochemistry* **31,** 2088–2092
44. Di Pietro, A., Penin, F., Julliard, H. J., Godinot, C., and Gautheron, D. C. (1988) *Biochem. Biophys. Res. Commun.* **152,** 1319–1325
45. Laemmli, U. K. (1970) *Nature* **227,** 680–685
46. Baracca, A., Degli Esposti, M., Parenti-Castelli, G., and Solaini, G. (1992) *Comp. Biochem. Physiol.* **101B,** 421–426
47. Lowry, O. H., Rosebrough, N. J., Farr, A. L., and Randall, R. J. (1951) *J. Biol. Chem.* **193,** 265–275
48. Strambini, G. B. (1983) *Biophys. J.* **43,** 127–130
49. Cioni, P., Piras, L., and Strambini, G. B. (1989) *Eur. J. Biochem.* **185,** 573–579
50. Strambini, G. B., and Gonnelli, M. (1986) *Biochemistry* **25,** 2471–2476
51. Boyer, P. D. (1989) *FASEB J.* **3,** 2164–2178
52. Drobinskaya, I. Y., Kozlov, I. A., Murataliev, M. B., and Vulfson, E. N. (1985) *FEBS Lett.* **182,** 419–424
53. Milgrom, Y. M., and Boyer, P. D. (1990) *Biochim. Biophys. Acta* **1020,** 43–48
54. Chernyak, B. V., and Cross, R. L. (1992) *Arch. Biochem. Biophys.* **295,** 247–252
55. Cross, R. L., Grubmeyer, C., and Penefsky, H. S. (1982) *J. Biol. Chem.* **257,** 12101–12105
56. Nalin, C. M., and Cross, R. L. (1982) *J. Biol. Chem.* **257,** 8055–8060
57. Kamp, G., and Juretschke, H. P. (1987) *Biochim. Biophys. Acta* **929,** 121–127
58. Kawano, Y., Tanokura, M., and Yamada, K. (1988) *J. Physiol.* (*Lond.*) **407,** 243–261
59. Senior, A. E. (1979) *J. Biol. Chem.* **254,** 11319–11322
60. Gromet-Elhanan, Z., and Khananshvili, D. (1984) *Biochemistry* **23,** 1022–1028
61. Aloise, P., Kagawa, Y., and Coleman, P. S. (1991) *J. Biol. Chem.* **266,** 10368–10376
62. Burgard, S., Nett, J. H., Sauer, H. E., Kagawa, Y., Schafer, H., Wise, J. G., Vogel, P. D., and Trommer, W. E. (1994) *J. Biol. Chem.* **269,** 17815–17819
63. Guelin, E., Chevallier, J., Rigoulet, M., Guerin, B., and Velours, J. (1993) *J. Biol. Chem.* **268,** 161–167
64. Joshi, S., and Burrows, R. (1990) *J. Biol. Chem.* **265,** 14518–14525
65. Pedersen, P. L., and Amzel, L. M. (1993) *J. Biol. Chem.* **268,** 9937–9940
66. Baracca, A., Amler, E., Solaini, G., Parenti-Castelli, G., Lenaz, G., and Houstek, J. (1989) *Biochim. Biophys. Acta* **796,** 77–84
67. Schlichting, I., Almo, S. C., Rapp, G., Wilson, K., Petratos, K., Lentfer, A., Wittinghofer, A., Kabsch, W., Pai, E. F., Petsko, G. A., and Goody, R. S. (1990) *Nature* **345,** 309–315
68. Gay, N. J., and Walker, J. E. (1983) *Nature* **301,** 262–264
69. Cleland, W. W., and Mildvan, A. S. (1979) in *Advances in inorganic Chemistry* (Eichorn, G., and Marzilli, L. G., eds) Vol. 1, pp. 163–191, Elsevier/North-Holland, Amsterdam
70. Frash, W. D., and Selman, B. R. (1982) *Biochemistry* **21,** 3636–3643
71. Dunn, S. D. (1982) *J. Biol. Chem.* **257,** 7354–7359
72. Gogol, E. P., Johnston, E., Aggeler, R., and Capaldi, R. A. (1990) *Proc. Natl. Acad. Sci. U. S. A.* **87,** 9585–9589
73. Richter, M. L., and McCarty, R. E. (1987) *J. Biol. Chem.* **262,** 15037–15040
74. Aggeler, R., Chicas-Cruz, K., Cai, S-X., Keana, J. F. W., and Capaldi, R. A. (1992) *Biochemistry* **31,** 2956–2961
75. Strambini, G. B., and Gonnelli, M. (1995) *J. Am. Chem. Soc.*, in press

Conformational Changes of the Mitochondrial F_1-ATPase ?-Subunit Induced by Nucleotide Binding as Observed by Phosphorescence Spectroscopy

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