Conformational Changes of the Mitochondrial F₁-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 F₁-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 Mg²⁺ 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 F₁ (Nd-F₁) and the F₁ preparation containing the three tightly bound nucleotides (F₁(2,1)). Since the effects observed are similar in both the F₁ 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-F₁. The effects on protein structure of nucleotide binding to either catalytic or noncatalytic sites have been distinguished by studying the phosphorescence emission of the F₁ complex prepared with the three noncatalytic sites filled and the three catalytic sites vacant (F₁(2,1)). Phosphorescence lifetime measurements on this F₁ 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 F₁(2,1) must exert the opposite and larger effect of enhancing the flexibility of the protein structure observed in both Nd-F₁ and F₁(2,1). The observation that enhanced flexibility of the protein occurs upon addition of adenine nucleotides to F₁(2,1) in the absence of Mg²⁺ 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: F₀, capable of proton transport across the membrane and the catalytic part; F₁, bound to F₀ through a "stalk" segment. Nonspecific binding sites for nucleotides and inhibitors of ATP synthase are present in both F₁ and F₀. F₁ contains one noncatalytic site and one catalytic site plus two noncatalytic sites; F₀ contains two noncatalytic sites.

The abbreviations used are: F₁(x,y), F₁ containing x mol of ANP at noncatalytic sites and y mol of ANP at catalytic sites per mol of enzyme; F₁ε, soluble part of the F-type H⁺-ATPases; Nd-F₁, nucleotide-depleted F₁; AMP-PNP, 5′-adenylyl-β,γ-imidodiphosphate; HPLC, high pressure liquid chromatography.

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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 F1 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 F1 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 F1, 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 F1.

MATERIALS AND METHODS

ATP, ADP, GTP, GDP, phosphonopyruvate, Hepes, Tris, and NADH were obtained from Sigma as were pyruvate kinase and lactate dehydrogenase in glycerol-containing buffers. Sephadex G-50, Sephacryl S-500, Blue Sepharose CL-6B, and protein standard marker proteins were obtained from Pharmacia Biotech Inc.

F1 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, 15 mg of protein (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 flow rate of 13 mg of the non-retained protein were concentrated to 15 mg/ml by ultrafiltration with a Diaflow XM-300 (Amicon) membrane. The concentrate was then chromatographed on Sephacryl S-500 (40 cm × 1 cm, inner diameter) in 25 mM Tris-Cl, pH 8 (42), which could result in a typical elution profile is shown in Fig. 1A. Fractions containing the 32–40 ml elution volume (9 mg of protein) were characterized 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 the least exponential term of F1 was observed (Fig. 1B). 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 F1, it is extremely important that no contaminating protein is present in the enzyme preparation to avoid Trp signals other than that of F1.

F2 was obtained from the above enzyme suspension by centrifugation, and the sedimented enzyme was dissolved at 4–6°C. The enzyme was not further purified since it is not required for the study of the ε-subunit (38). 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 phosphors were generated by a Cernax xenon lamp (LX 150UV, ILC Technology), and the wavelength was set with a 250-nm grating monochromator (J obin-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 (λex = 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 μJ. 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 high speed chopper and 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 computer system (ISC-16, RC Eletronica) 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 the least exponential term of F1 was observed (Fig. 1B). 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 F1, it is extremely important that no contaminating protein is present in the enzyme preparation to avoid Trp signals other than that of F1. 

RESULTS

Nucleotide Effects on Nd-F1. Phosphorescence Decay—Fig. 2 shows the selected examples of phosphorescence intensity decay of Nd-F1 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-F1 with adenine nucleotides at 1 mM in the presence of magnesium changes the intrinsic phosphorescence decay parameters of Nd-F1. 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-F1 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).
ADP has an effect opposite that of ATP; it enhances the protein rigidity since the intrinsic $\tau_{av}$ of the protein increases from 2.8 of Nd-F$_1$ to 3.4 ms upon nucleotide binding.

The opposite effect induced on the $\epsilon$-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 $\epsilon$-subunit. This would be consistent

**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 $\alpha$-lactalbumin (14.4 kDa); lanes B-D, 4, 6, and 10 $\mu$g of Nd-F$_1$, respectively; lanes E and F, 3 and 9 $\mu$g of F$_1$(3,0), respectively; lanes G and H, 15 and 20 $\mu$g of F$_1$(2,1), respectively. The positions of the F$_1$ subunits are indicated at the right side.
affected 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 F1 have to be occupied to induce ε-subunit conformational changes, preparations of F1 at different levels of nucleotide occupancy of sites have been studied.

F1(2,1) Phosphorescence Decay Changes Induced by Nucleotides—Preliminarily, we obtained the phosphorescence spectra of the F1(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-F1 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 F1(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 MgSO4, 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 \( \tau_{av} \) to 4.6 ms (Table I). Thus, the results are similar to those observed on Nd-F1, 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 (Mg2+ 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 F1 conformation. Thus, experiments designed to determine whether inorganic phosphate added together with ADP might have the capability to influence the ε-subunit conformation

**Table I**

| F1 form | Ligand | Concentration | Phosphorescence decay |
|---------|--------|---------------|----------------------|
| NdF1   | mM     | ms            | \( \tau_{av} \)     |
| NdF1   | Mg-ATP | 1             | 1.4 0.84 10 0.16 2.8 |
| NdF1   | Mg-ATP | 5·10^{-3}     | 1.2 0.86 7.4 0.14 2.1 |
| NdF1   | Mg-ADP | 1             | 1.9 0.78 8.6 0.22 3.4 |
| NdF1   | Mg-ADP | 3·7·10^{-3}   | 1.3 0.75 6.1 0.25 2.5 |
| NdF1   | Mg-ATP | 3·7·10^{-3}   | 1.3 0.85 11 0.15 2.7 |
| F1(2,1)| Mg-ATP | 1             | 2.7 0.71 8.2 0.29 4.3 |
| F1(2,1)| Mg-ATP | 1             | 3.5 0.44 4.8 0.56 3.3 |
| F1(2,1)| Mg-GTP | 5·10^{-1}     | 1.6 0.54 7.4 0.46 3.5 |
| F1(2,1)| Mg-ADP | 1             | 3.3 0.67 7.4 0.34 4.6 |
| F1(2,1)| Mg-ADP + P(i) | 1 | 3.2 0.58 7.0 0.42 4.8 |
| F1(3,0)| Mg-ATP | 1             | 1.0 0.8 9.8 0.28 2.5 |
| F1(3,0)| Mg-ATP | 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 F1-ATPase complex may exist into two different conformations, Es and Eh, favored by ADP and ATP binding, respectively.

When Nd-F1 is incubated with stoichiometric amounts of ADP in the presence of Mg2+, 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-F1. 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-F1 (i.e. conditions for unisite catalysis as in Ref. 55) does not
produces a significant tightening of the e-subunit N-terminal domain, which might be explained if one considers that Mg$^{2+}$ ions likely overwhelm the tightening of the same domain induced by nucleotide binding. The enhanced rigidity associated with the filling of the exchangeable noncatalytic site and the enhanced rigidity associated to the Mg$^{2+}$-subunit conformation (Table II).

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 enhanced flexibility associated with the filling of the exchangeable noncatalytic site induces an increased flexibility of the e-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 e-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 e-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 e-subunit conformation (Table I).

Addition of ATP in the presence of 2 mM EDTA reduces the $\tau_{av}$ 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 $\tau_{av}$, 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 e-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 $\tau_{av}$ 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 $\epsilon$-subunit conformation.

**DISCUSSION**

The $\epsilon$-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 $\epsilon$-subunit location is not available, most of the F$_1$ and F$_0$ models show the polypeptide located at the interior of the $\alpha$-$\beta$-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 $\epsilon$-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 $\epsilon$-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 $\epsilon$-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 unistochiometric 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 unistochiometric 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 $\epsilon$-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 $\epsilon$-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, F$_1$ can not influence the ADP-F$_1$ structure as probed at the $\epsilon$-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-GTP 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 $\epsilon$-subunit, depending on the particular type of stereoisomer bound.

If the $\epsilon$-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 $\epsilon$-subunit might be involved in the propagation of signals to deeper regions of the F$_0$-F$_1$ complex.

Finally, observations of certain similarities between the present study and those carried out on F$_1$ from other sources should be noted. Evidence for a correlation between occupation of the nucleotide binding sites, catalysis, and conformational changes of the $\epsilon$-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 $\alpha_2\beta_2\gamma$ 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$_1$ $\epsilon$-subunit, which has no counterpart in other species, might share with the $\epsilon$-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 $\epsilon$-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 $\epsilon$-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 $\epsilon$-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 e-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. Hatlel, Y. (1993) Eur. J. Biochem. 218, 759–767
5. Issartel, J. P., Dupuis, A., Garin, J., Lunardi, J., Michél, L., and Vignais, P. V. (1992) Experientia 48, 351–362
6. Walker, J. E., Feamley, 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., Jilliard, H. J., Godinot, C., and Gautheron, D. C. (1988) Biochim. Biophys. Res. Commun. 152, 1319–1325
12. Lauter, R., Abrahams, J. P., Van Raaij, M. J., Todd, R. J., Lundqvist, T., Buchanan, S. K., Lenaz, G. A. W., and Walker, J. E. (1993) J. Mol. Biol. 229, 787–790
13. Milgrom, Y. M., and Boyer, P. D. (1990) Comp. Biochem. Physiol. 101B, 421–426
14. Lowry, O. H., Rosebrough, N. J., Farr, A. L., and Randall, R. J. (1951) J. Biol. Chem. 193, 265–275
15. Richter, M. L., Snyder, B., McCarty, R. E., and Hammes, G. G. (1985) J. Biol. Chem. 260, 21571–21578
16. Guelin, E., Chevallier, J., Rigoulet, M., Guerin, B., and Velours, J. (1993) Eur. J. Biochem. 218, 177–231
17. Gogol, E. P., Johnston, E., Aggeler, R., and Capaldi, R. A. (1990) J. Mol. Liq. 52, 2956–2961
18. Cleland, W. W., and Mildvan, A. S. (1979) in Experientia, 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. Hatlel, Y. (1993) Eur. J. Biochem. 218, 759–767
5. Issartel, J. P., Dupuis, A., Garin, J., Lunardi, J., Michél, L., and Vignais, P. V. (1992) Experientia 48, 351–362
6. Walker, J. E., Feamley, 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., Jilliard, H. J., Godinot, C., and Gautheron, D. C. (1988) Biochim. Biophys. Res. Commun. 152, 1319–1325
12. Lauter, R., Abrahams, J. P., Van Raaij, M. J., Todd, R. J., Lundqvist, T., Buchanan, S. K., Lenaz, G. A. W., and Walker, J. E. (1993) J. Mol. Biol. 229, 787–790
13. Milgrom, Y. M., and Boyer, P. D. (1990) Comp. Biochem. Physiol. 101B, 421–426
14. Lowry, O. H., Rosebrough, N. J., Farr, A. L., and Randall, R. J. (1951) J. Biol. Chem. 193, 265–275
15. Richter, M. L., Snyder, B., McCarty, R. E., and Hammes, G. G. (1985) J. Biol. Chem. 260, 21571–21578
16. Guelin, E., Chevallier, J., Rigoulet, M., Guerin, B., and Véours, J. (1993) J. Biol. Chem. 268, 161–167
17. Joshi, S., and Burrows, R. (1990) J. Biol. Chem. 265, 14518–14525
18. Pedersen, P. L., and Amzel, L. M. (1993) J. Biol. Chem. 268, 9937–9940
19. Baracca, A., Amter, E., Solaini, G., Parenti-Castelli, G., Lenaz, G., and Housetek, J. (1989) Biochim. Biophys. Acta 796, 77–84
20. Schlichting, I., Almo, S. C., Rapp, G., Wilson, K., Petratos, K., Lentfer, A., Wittinghofer, A., Kabsch, W., Pal, E. F., Petsko, G. A., and Goody, R. S. (1990) Nature 345, 309–315
21. Gay, N. J., and Walker, J. E. (1983) Nature 301, 262–264
22. 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
23. Frash, W. D., and Selman, B. R. (1982) Biochemistry 21, 3636–3643
24. Dunn, S. D. (1982) J. Biol. Chem. 257, 7354–7359
25. Gogol, E. P., Johnston, E., Aggeler, R., and Capaldi, R. A. (1990) Proc. Natl. Acad. Sci. U. S. A. 87, 9585–9589
26. Richter, M. L., and McCarty, R. E. (1987) J. Biol. Chem. 262, 15037–15040
27. Aggeler, R., Chicas-Chur, K., Cai, S.-X., Kenna, J. F. W., and Capaldi, R. A. (1992) Biochemistry 31, 2956–2961
28. Strambini, G. B., and Gonnelli, M. (1995) J. Am. Chem. Soc., in press