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A STUDY OF THE MITOCHONDRIAL F_1-ATPase TRYPTOPHAN PHOSPHORESCENCE AT 273 K

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SUMMARY: The bovine heart mitochondrial F_1-ATPase complex exhibits an intrinsic tryptophan phosphorescence that can be used to monitor structural changes of the ε-subunit. The phosphorescence decay rate of F_1 containing the tightly bound nucleotides increases upon addition of adenine nucleoside triphosphate in the presence of magnesium. The average phosphorescence lifetime of this enzyme preparation decreases from 10.2 to 7.8 ms upon Mg-ATP addition. Since increasing phosphorescence decay rate is related to increasing flexibility of proteins, Mg-ATP added to the F_1-ATPase complex can enhance the flexibility of the protein structure surrounding the chromophore. Experiments carried out on F_1 prepared with the three noncatalytic sites filled and the three catalytic sites vacant show a significant increase of the phosphorescence lifetime from 6.4 ms to 7.6 ms upon Mg-ATP addition. These results suggest that the mitochondrial F_1-ATPase ε-subunit conformation senses differently the nucleoside triphosphate binding to catalytic or noncatalytic sites.

The isolated ATPase component of the mitochondrial ATP synthase (F_1) has an $\alpha_3\beta_3\gamma\delta\varepsilon$ subunit composition. The smallest of these is the ε-subunit (1-3). In the enzyme isolated from bovine heart mitochondria this is a polypeptide of unknown function 50 amino acid long, containing a single Trp in the N-terminal segment at position 4 (4). In order to explore on the possible role of the subunit, we have recently used the phosphorescence emission of the sole tryptophan residue of the mitochondrial F_1 complex as an internal probe of the ε-subunit (5). In particular, from phosphorescence decay analysis we could calculate the lifetime parameter that is related to the nature and flexibility of the

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The abbreviations used are: F_1, soluble part of the F-type mitochondrial H^+-ATPase; $F_1[x,y]$, F_1 containing x mol of ANP at noncatalytic sites and y mol of ANP at catalytic sites per mol of enzyme; ANP, adenine nucleotide.

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chromophore environment, providing unique informations of the ε-subunit conformation in situ as a consequence of reloading with Mg-ATP the nucleotide-depleted enzyme.

Since F_1 contains a total of six nucleotide binding sites we carried out several experiments in order to find possible relationships between filling different sites and conformational changes of the ε-subunit. The nucleotide binding sites are located at interfaces between the two main subunits α and β (6-8) and can be distinguished as catalytic or noncatalytic according to their ability to exchange bound ligand rapidly during hydrolysis of Mg-ATP (9,10). 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 (11,12) showed that binding of ATP to these sites induced maximal steady-state rates of hydrolysis of nucleoside triphosphates. On the basis of kinetic experiments, the above authors and others support the idea that noncatalytic sites have a regulatory function (13-15). Therefore, if one could find a relationship between one or the other of the two classes of nucleotide binding sites with the ε-subunit, the functional role of the polypeptide should likely be associated with the function of that class of sites. In the present study, the phosphorescence lifetime of the mitochondrial F_1 Trp has revealed conformational changes of the ε-subunit in response to the binding of Mg-ATP to catalytic and noncatalytic nucleotide binding sites.

MATERIALS AND METHODS

The F_1-ATPase complex was prepared from bovine heart mitochondrial (16) following a modified Penefsky's procedure (17). F_1 was released from sonicated submitochondrial particles (18) and passed through a set of chromatography columns (manuscript in preparation). The enzyme solution (250 mM sucrose, 2 mM EDTA, 4 mM ATP and 50 mM Tris/Cl, pH 8) was stored at 5 °C as a suspension adding an equal volume of saturated ammonium sulfate solution (pH 8) and the enzyme activity is stable for several weeks.

In order to obtain an enzyme preparation characterized by two endogenous nucleotides bound at noncatalytic sites and one bound at a catalytic site, $F_1[2,1]$ (19), the enzyme suspension was centrifuged at 44000 g x 15' and the enzyme pellet, dissolved at 4-6 μM in a buffer containing 150 mM sucrose, 1 mM $MgSO_4$, 10 mM K^+-Hepes pH 8, was desalted by passage through a sephadex G-50 centrifuge column (20) equilibrated in the same buffer. Prior to being dissolved the F_1 pellet was gently rinsed with small volumes of 2.8 M ammonium sulfate, 10 mM Tris/SO_4, pH 8 to remove the nucleotide excess. $F_1[3,0]$, which indicates that the only three noncatalytic sites are occupied by endogenous nucleotides, was prepared from the stored enzyme according to the procedure described above for $F_1[2,1]$ then the protein was passed through sephadex G-50 centrifuge columns following a procedure based on the displacement of nucleotides from the catalytic sites by pyrophosphate (19).

The nucleotide content of each enzyme preparation was determined by reverse-phase HPLC, following nucleotide extraction according to Di Pietro et al. (21).

The ATPase activity was measured 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 (22) and the specific activity of the enzyme was 60-90 U/mg protein at 20 °C.

Protein concentration of enzyme solutions was determined by the method of Lowry et al. (23) and protein concentration of mitochondria and submitochondrial particles was determined by the biuret method (24). No correction for the contribution of haem groups to the 540 nm absorption was made.

For phosphorescence studies the apparatus employed was a home-made phosphorimeter described elsewhere (25); the experiments were carried out at 273 K and the samples to be investigated were prepared as reported in Solaini et al. (5).

RESULTS AND DISCUSSION

Phosphorescence emission spectroscopy is a useful tool for investigating structure-function relationships in biological systems. Not only are structural informations obtainable, the technique is also very sensitive to conformational changes in the environment of the chromophore, in particular an inverse relationship between the phosphorescence lifetime and the flexibility of the polypeptide chain around the chromophore has been reported (26). Previously, through the investigations of the intrinsic phosphorescence of the mitochondrial F_1-ATPase complex under static and dynamic conditions, we showed that adding Mg-ATP to the nucleotide depleted enzyme at 273 K a large conformational change and an increase of flexibility of the protein surrounding the N-terminal domain of the ε-subunit was induced (5). In this communication we report on the effects elicited on the same protein domain when the enzyme having different nucleotide configurations is incubated with Mg-ATP.

The mitochondrial F_1-ATPase complex is usually stored at 5°C as a suspension in a 50 % saturated ammonium sulfate solution containing 2 mM ATP (17). Enzyme to be used for the present experiments is centrifuged through a 1 ml Sephadex G-50 column (19) as detailed under materials and methods. This gives preparations containing nearly three mol of tightly bound nucleotides (ATP+ADP) per mol of enzyme in good agreement with the literature (27,28). According to the criteria defined by Kironde and Cross (1986) the three nucleotides occupy two noncatalytic sites and a single catalytic site and the enzyme is refered to as F_1[2,1].

Figure 1 shows the phosphorescence decay of F_1[2,1] at 273 K upon excitation of the protein at 292 nm. Under all conditions examined, the phosphorescence decay is clearly heterogeneous. Since there is only one Trp in the macromolecule the heterogeneity must derive from a nonuniform flexibility of its environment, a variability that implies a multiplicity of coexisting long-lived conformations of the ε-subunit (5).

Incubation of F_1[2,1] with 1 mM Mg-ATP at 273 K in 75 mM sucrose, 25 mM KH_2PO_4, 1 mM $MgSO_4$, 5 mM K^+-Hepes, pH 8 and 50% glycerol induces consistent changes of the phosphorescence decay. Table I shows the phosphorescence decay

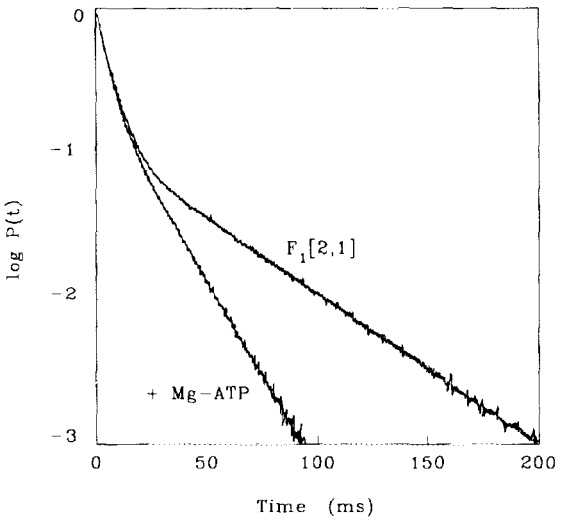

Fig.1. Decay of tryptophan phosphorescence of F_1[2,1] in presence or absence of 1 mM Mg-ATP at 273 K.
Experimental details are described in the legend to Table I.

parameters of a typical experiment. The average phosphorescence lifetime (τ_{av}) of the enzyme in the presence of the nucleotide decreases over 20% from 10.2 to 7.8. This indicates an increased flexibility of the chromophore environment upon occupancy of the

Table I

Nucleotide Binding Effects on F_1[2,1] and F_1[3,0] Phosphorescence Decay

F_1-form	Ligand	Phosphorescence decay				
		τ_1 (ms)	α_1	τ_2 (ms)	α_2	τ_{av}* (ms)
F_1[2,1]	-	5.9	0.88	41.6	0.12	10.2
F_1[2,1]	ATP	4.6	0.73	16.6	0.27	7.8
F_1[3,0]	-	5.6	0.95	22.6	0.05	6.4
F_1[3,0]	ATP	5.5	0.90	26.8	0.1	7.6

F_1[2,1] (1.5 μM) and F_1[3,0] (1μM) were in 75 mM sucrose, 0.5 mM $MgSO_4$, 25 mM KH_2PO_4, 5mM Hepes and 50% glycerol, 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. Phosphorescence decays (λ_{ex}= 292 nm) were measured at 273 K.

*$\tau_{av} = \tau_1\alpha_1 + \tau_2\alpha_2$.

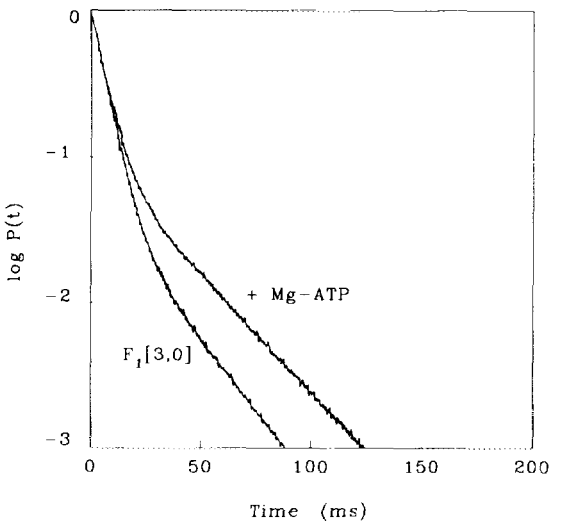

Fig.2. Decay of tryptophan phosphorescence of $F_1[3,0]$ in presence or absence of 1 mM Mg-ATP at 273 K.
Experimental details are described in the legend to Table I.

vacant nucleotide binding sites. The effect is attributed to the filling with Mg-ATP of either or both the catalytic and the noncatalytic site(s) left vacant in the $F_1[2,1]$ form.

The F_1-ATPase complex can be prepared with the nucleotide configuration characterized by the three filled noncatalytic sites and three vacant catalytic sites (19). This enzyme preparation shows an intrinsic phosphorescence decay which can be modulated by adenine nucleotide triphosphate binding (Fig. 2). Kinetic parameters of a typical phosphorescence decay are listed in Table I. Incubating at 273 K the enzyme along with Mg-ATP can enhance the intrinsic phosphorescence average lifetime from 6.4 ms to 7.6 ms indicating that filling of the catalytic sites with Mg-ATP results in tightening of the ε-subunit conformation. All together, the results suggest that the effects observed on addition of nucleotides to $F_1[2,1]$ are the sum of two distinct events: the filling of the vacant noncatalytic site that induces an increased flexibility of the ε-subunit N-terminal domain conformation which likely overwhelms the tightening of the same domain induced on filling the catalytic binding sites.

Assessment of the validity of this hypothesis requires first to provide direct evidence that the binding of nucleotide to the vacant noncatalytic site of $F_1[2,1]$ affects the ε-subunit conformation and second, to examine the effects of nucleotides binding at a temperature above 18 °C and in a medium characterized by lower density and viscosity. In fact under the conditions used the k_{cat} and the K_m of the enzyme for ATP result respectively 10-fold lower and 4-fold higher with respect to those evaluated at temperature above 18°C (29);

besides the mitochondrial F_1-ATPase is characterized by two different conformations above and below 18°C (22). Work is in progress in order to pursue this aim.

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