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Nucleotide Binding Drives Conformational Changes in the Isolated α and β Subunits of the F_1-ATPase from *Escherichia coli*

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The modeling of the rotatory mechanism performed by the F_1-ATPase complex during ATP synthesis shows that the β, but not the α subunit, undergoes large conformational changes that depend on the occupancy of the catalytic site. Here we determined by fluorescence spectroscopy the changes in tertiary structure and hydrophobic exposed area of the isolated α and β subunits of the F_1-ATPase complex from *Escherichia coli* upon adenine nucleotide binding. The results show that in the absence of intersubunit contacts, the two subunits exhibit markedly similar conformational movements.

Key Words: **F_1-ATPase; ligand binding; intrinsic fluorescence; 1-anilino-8-naphthalene sulfonate.**

During oxidative or photosynthetic phosphorylation, the F_0F_1-ATPsynthase/ATPase complex of energy transducing membranes catalyzes ATP synthesis from $ADP \cdot Mg^{2+}$ and Pi with the energy of H^+ or Na^+ gradients (1). The enzyme can also hydrolyze ATP and generate electrochemical gradients. This complex is arranged in two domains (1, 2): F_0, which functions as an ion pathway across the membranes and a hydrophilic domain, F_1, which contains the catalytic machinery. F_1 can be detached from the membranes as a soluble ATPase; it is formed by five different subunits α_3, β_3, γ, δ, and ε. The α and β subunits have similar amino acid sequences and three-dimensional structures. The two have a nucleotide binding site, however, only the binding sites in the β subunit are catalytic (2, 3). The crystal structure of mitochondrial F_1 shows that the three β subunits are asymmetric and their conformation is related to the nucleotide that occupies the binding site. The conformation of F_1 is in consonance with the hypothesis that F_1 carries out catalysis through an alternating site mechanism (3, 4).

In regard to the conformational changes that the α and β subunits undergo in the whole enzyme complex, Wang and Oster (5, 6) showed that the upper and lower domains of β subunit undergo a hinge motion of about 30°. The α subunit did not exhibit significant movements (5). However, sedimentation analysis of the α subunit from *Escherichia coli* showed that ATP binding induces a large conformational change (7). Likewise, ATP binding increased the electrophoretic mobility of the α subunit and enhanced its resistance to trypsin proteolysis (8, 9). The isolated β subunit also exhibits conformational change upon ADP or ATP binding as shown by: an increase in its affinity for aurovertin (10), quenching of ANS (1-anilino-8-naphthalene sulfonate) fluorescence (11, 12), an increased resistance to trypsin (8). In other studies it was suggested that after modification with 3′-*O*-(4-benzoyl)benzoyl-ATP the β subunit undergoes a hinge motion (13, 14).

The latter observations indicate that nucleotide binding is linked to conformational changes of both subunits. However, they do not provide information of their extent, nor of the regions of the protein that undergoes such conformational changes. Therefore, we determined the effect of nucleotide binding on the intrinsic fluorescence of the two subunits in order to ascertain which regions of the proteins undergo such changes. We also determined if the solvent exposed hydrophobic regions of the subunits exhibit variations upon adenine nucleotide binding.

MATERIALS AND METHODS

Cell growth and protein expression. E. coli cells (strain DK8), containing the pBWA1 or pUWD1 plasmid expressing α or β subunit, respectively (15, 16) were grown and lysed as described (15).

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Purification of the recombinant α subunit. The lysate was spun at 100,000*g* for 1 h at 4°C. The supernatant was taken to 40% saturation with ammonium sulfate and incubated overnight; thereafter it was centrifuged at 100,000*g* for 1 h at 4°C; the supernatant was precipitated with ammonium sulfate (70% saturation) for 4 h and centrifuged at 100,000*g* for 20 min at 4°C. The pellet was dissolved in 50 mM Tris/0.5 mM EDTA pH 7.6 (buffer A) that had 0.5 M NaCl. Thereafter, the solution was loaded on a phenyl-Sepharose Cl-4B column (2.5 × 16 cm) equilibrated with 75 mM Tris/0.5 mM EDTA/0.05 mM DTT/0.5 M NaCl, pH 7.6. The column was washed with the same buffer without NaCl, and the protein was eluted with water. The fractions containing the α subunit were pooled and loaded on a hydroxylapatite-HTP Biogel column (1.5 × 20 cm) equilibrated with buffer A. The protein was eluted with a linear gradient of 0-250 mM sodium phosphate in the same buffer; desalted in a phenyl-Sepharose Cl-4B column in the conditions mentioned above and further purified by anion exchange chromatography in a mono Q HR 10/10 column equilibrated with buffer A in a FPLC system. The protein was eluted with a linear gradient of 0-5 M NaCl in buffer A. The fractions were analyzed by SDS/PAGE and Coomassie blue staining. Fractions containing α subunit were pooled and stored in 10% glycerol in buffer A at −75°C. Typically, 1 L of cell culture yielded around 2.5 mg of α subunit (MW 55 kDa).

Purification of the recombinant β subunit. The lysate was spun at 100,000g for 1 h at 4°C. The supernatant was precipitated overnight with ammonium sulfate (45%) and centrifuged at 10,000g for 20 min at 4°C. The supernatant was precipitated with ammonium sulfate (75% saturation) for 4 h and centrifuged at 10,000*g* for 20 min at 4°C. The pellet was resuspended in 10 mL 50 mM succinate-Tris/0.5 mM EDTA/0.05 mM β-mercaptoethanol/0.5 M NaCl, pH 7.6. Thereafter, the solution was loaded on a phenyl-Sepharose Cl-4B column (2.5 × 16 cm) equilibrated with buffer A containing 0.5 M NaCl and washed with 200 mL buffer A. The protein was eluted with 0.5 mM EDTA/0.05 mM β-mercaptoethanol pH 7.6. The fractions containing β subunit were pooled and loaded on the mono Q HR 10/10 column equilibrated with buffer A. The protein was eluted with a gradient of NaCl in the same buffer (0–0.2 M). The β subunit eluted at approximately 175 mM NaCl. The fractions with β subunit, as identified by SDS-PAGE, were pooled and stored in 10% glycerol/0.5 mM β-mercaptoethanol in buffer A at −75°C. Typically, 1 L of culture yielded 4.5 mg of β subunit (MW 50.2 kDa).

Fluorescence measurements. The changes in fluorescence were monitored using a ISS PC1 Photon Counting Spectrofluorometer (ISS, Champaign, IL) with the cell compartment thermoregulated at 25°C. Intrinsic fluorescence was measured at excitation wavelengths of 280 and 295 nm, with a 8.0 nm bandwidth. Emission spectra were collected with a 16 nm bandwidth from 300 to 450 nm (excitation at 280 nm) or 314–414 nm (excitation at 295 nm). All samples (0.5 μM subunits) were equilibrated for 10 min in 50 mM Tris-HCl with or without the ligands indicated under Results.

ANS fluorescence was measured at an excitation wavelength of 356 nm, with an 8 nm bandwidth, in the emission range of 400-560 nm (16.0 nm bandwidth). All samples (1.0 μM subunits) were equilibrated for 10 min in 50 mM Tris-HCl with 7.6 μM ANS (11) with or without the ligand(s).

Other procedures. Protein concentration was quantified according to Smith (17). Polyacrylamide gel electrophoresis was carried out according to (18). The gels were stained with Coomassie blue. The amount of [^{32}Pi] formed from [γ-^{32}Pi]ATP was used to assay hydrolysis as described (19).

RESULTS

The ATPase activity of the β subunit was 0.4 ± 0.03 nmol mg^{-1} min^{-1}; the α subunit did not exhibit activity. For measurements of the effect of nucleotides on the intrinsic fluorescence of the two subunits, the excitation wavelengths of 280 and 295 nm were used in order to sense the change in tyrosine and tryptophan environments, respectively. After nucleotide incubation, the spectral center mass and λ_{max} of the two subunits were almost identical. However, there were differences in fluorescence intensity of the α and β subunits when adenine nucleotides were added (Fig. 1).

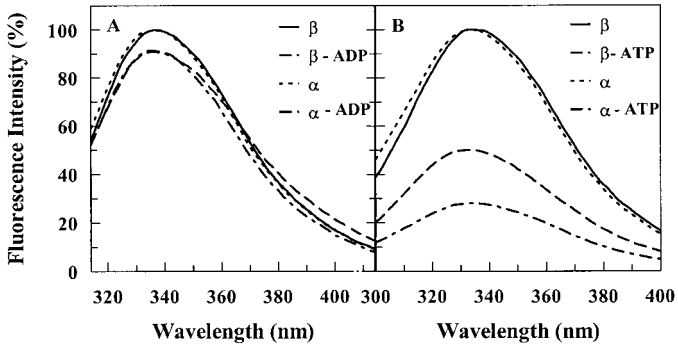

FIG. 1. Intrinsic fluorescence emission spectra for α and β subunits. The α and β subunits (50 μg mL^{-1}) were incubated in the presence of ATP or ADP (1 mM). Thereafter emission spectra were recorded at a excitation wavelength of 295 nm (A) or 280 nm (B).

Tryptophan fluorescence after nucleotide binding. At an excitation wavelength of 295 nm, notwithstanding the presence or absence of Mg^{2+}, the intrinsic fluorescence of the α subunit was increased, albeit slightly, by ATP, ADP, and AMPPNP (Table 1). ATP did not modify the intrinsic fluorescence of the β subunit, but ADP and AMPPNP produced a small decrease in the signal. The three-dimensional structure of both α and β *E. coli* subunits were estimated trough the Swiss model protocol (20). The studies showed that in the two subunits the only tryptophan is more than 30 Å away from the binding site.

Tyrosine fluorescence after nucleotide binding. The α and β subunits have 16 and 15 tyrosines, respectively. The modeling of the subunits (20) showed that there is a region rich in tyrosines near the adenine nucleotide binding sites. At an excitation wavelength of 280 nm, with and without Mg^{2+}, ATP brought about a 64% quenching of fluorescence of the β subunit (Table 2). In the modeling of F1 complex by Wang and Oster (5, 6), no significant movements of the α subunit were detected. However, we observed that ATP induced a 70% decrease of intrinsic fluorescence in the α subunit regardless of the presence or absence of Mg^{2+} (Table 2).

ADP, with and without Mg^{2+} also induced significant quenching of fluorescence of the α and β subunits, but this was lower than that observed with ATP (Table 2). In contrast to ADP and ATP, AMPPNP did not produce fluorescence quenching. This could indicate that this analog does not bind to the isolated subunits, or that its binding does not induce a conformational change

TABLE 1

Changes in Tryptophane Fluorescence Intensity after Nucleotide Binding

Condition	α	β
ATP	28 ± 2	36 ± 14
ATP + Mg^{2+}	32 ± 3	32 ± 2
ADP	42 ± 10	40 ± 4
ADP + Mg^{2+}	42 ± 3	41 ± 1
AMPPNP	98 ± 5	91 ± 2
AMPPNP + Mg^{2+}	99 ± 2	93 ± 4
Mg^{2+}	123 ± 11	98 ± 9

Note. Samples were incubated with ATP, ADP, or AMPPNP (1 mM) with or without Mg^{2+} (1 mM). Thereafter emission was collected after excitation at 295 nm. The fluorescence intensity at λ_{max} (336 nm for both subunits) in the absence of ligands was assigned a value of 100%.

TABLE 2

Changes in Tyrosine Fluorescence Intensity after Nucleotide Binding

Condition	α	β
ATP	99 ± 11	107 ± 11
ATP + Mg^{2+}	108 ± 1	95 ± 3
ADP	104 ± 12	83 ± 8
ADP + Mg^{2+}	106 ± 4	89 ± 4
AMPPNP	108 ± 4	93 ± 7
AMPPNP + Mg^{2+}	112 ± 1	88 ± 3
Mg^{2+}	121 ± 11	87 ± 1

Note. Samples were incubated in the presence of ATP, ADP, or AMPPNP (1 mM) with or without Mg^{2+} (1 mM). Thereafter emission was collected after excitation at 280 nm. The fluorescence intensity at λ_{max} (334 or 336 nm for α and β subunits, respectively) in the absence of ligands is 100%.

similar to that induced by ADP and ATP. In Table 2, it may also be noticed that Mg^{2+} per se induces a 20% increase in the intrinsic fluorescence of the α subunit. Mg^{2+} also quenched the fluorescence of the β subunit, but to a lower extent. The former observation could be explained by the charge screening in the α subunit due to the binding of Mg^{2+}.

Changes in ANS fluorescence after nucleotide binding. The fluorescence intensity of ANS increased 4 to 5 times in the presence of 50 μg of the α or β subunits (data not shown). Since the binding of ANS to hydrophobic segments is accompanied by an increase in its fluorescence quantum yield, the data indicate that the isolated subunits have some hydrophobic regions exposed to the solvent. Thus, ANS was used to probe if these regions undergo alterations upon nucleotide binding. In the α subunit, without Mg^{2+}, ATP produced a 20% quenching of ANS fluorescence (Table 3). In presence of Mg^{2+}, ATP quenched fluorescence to the same extent, but ADP induced a higher quenching (Table 3). Thus, it would seem that the hydrophobic solvent exposed regions of the α subunit respond differently to the binding of ADP or ATP. It should be noted that ANS fluorescence quenching was lower in the β subunit when ADP was bound. AMPPNP did not affect the ANS fluorescence in either case.

The effect of nucleotides binding on the β subunit differs from that in the α subunit. In the former with and without Mg^{2+}, the extent of ANS quenching was much lower than in the α subunit.

DISCUSSION

It is well documented that all proteins undergo conformational changes during catalysis or upon ligand binding. The changes may be small as with lysozyme (21, 22) or large as with myosin (23, 24). Allosteric control is also mediated via conformational changes that modify the shape and volume of the enzyme and its biological function. In oligomeric enzymes, subunit interactions are central in the conformational changes that such proteins undergo. Hence, the characteristics of the monomers of multimeric enzymes are now being extensively studied in order to understand how subunit assembly modifies their behavior, and their function in the whole enzyme complexes. In general, monomers or monomeric intermediates of the folding and assembly pathway are ill equipped to express full catalytic function (19). This indicates that for many oligomers, the contacts between subunits are fundamental for expression of biological function. Indeed, this is the case of the β subunits of the F_1-ATPase. In this enzyme, the activity of the monomers is orders of magnitude lower than that observed in whole complex. Thus, the assembly of the subunits confers to the subunits a high catalytic efficiency. Mechanistically, this is mediated via a highly cooperative alternating site mechanism (3) in which the γ subunit rotates within the core of the α and β subunits (6).

TABLE 3

Changes in ANS Fluorescence after Nucleotide Binding

Condition	α	β
ATP	78 ± 8	80 ± 5
ATP + Mg^{2+}	83 ± 8	86 ± 3
ADP	57 ± 6	81 ± 4
ADP + Mg^{2+}	67 ± 7	86 ± 1
AMPPNP	75 ± 8	81 ± 2
AMPPNP + Mg^{2+}	84 ± 8	77 ± 2
Mg^{2+}	84 ± 8	86 ± 8

Note. The α and β subunits (50 μg mL^{-1}) were incubated in the presence of ligands (1 mM) with a saturating concentration of ANS (7.6 μM). The difference in ANS fluorescence intensity (excitation at 356 nm, emission at 480 nm) in the presence and absence of subunits is 100%.

The molecular dynamics studies of Wang and Oster (5), show that the β subunit has a hinge motion that involves movements of the upper and lower domains. In this model, the α subunit does not exhibit large conformational movements. However, we found that both subunits undergo significant changes in intrinsic fluorescence of tyrosines upon nucleotide binding, and that the sole tryptophan did not exhibit important alterations. These observations may be rationalized according to the location of the tyrosines and tryptophan residues in the three-dimensional structure of both subunits. Seven tyrosines are close to the binding site, and therefore, it is likely that these particular residues account for the observed modifications of intrinsic fluorescence. It is also noted that the latter changes involved a modification of fluorescence intensity without a shift in λ_{max}, implying that after nucleotide binding, there is an increase in energy transfer between tyrosine residues.

The lack of response of tryptophan fluorescence in the α and β subunits may indicate that the events at the catalytic site are not transmitted to that region of the enzyme (which is more than 30 Å away), or that this residue is already exposed to the solvent. However, the data of ANS fluorescence, which monitors non-buried hydrophobic areas, showed that the events at the catalytic site are transmitted to the surface of the subunits. Therefore, the binding of nucleotides induces extensive conformational changes in both subunits.

Since the two subunits have nearly the same number of tyrosine residues distributed more or less equally in the same regions, it may not be surprising that both subunits exhibit almost the same response to nucleotide binding, and apparently very similar conformational changes. Therefore, these observations imply that, unless their assembly into the enzyme complex, the isolated and soluble α and β subunits exhibit similar conformational changes upon adenine nucleotide binding.

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