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Isolated noncatalytic and catalytic subunits of F_1-ATPase exhibit similar, albeit not identical, energetic strategies for recognizing adenosine nucleotides

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ABSTRACT

The function of F_1-ATPase relies critically on the intrinsic ability of its catalytic and noncatalytic subunits to interact with nucleotides. Therefore, the study of isolated subunits represents an opportunity to dissect elementary energetic contributions that drive the enzyme's rotary mechanism. In this study we have calorimetrically characterized the association of adenosine nucleotides to the isolated noncatalytic α-subunit. The resulting recognition behavior was compared with that previously reported for the isolated catalytic β-subunit (N.O. Pulido, G. Salcedo, G. Pérez-Hernández, C. José-Núñez, A. Velázquez-Campoy, E. García-Hernández, Energetic effects of magnesium in the recognition of adenosine nucleotides by the F_1-ATPase β subunit, Biochemistry 49 (2010) 5258–5268). The two subunits exhibit nucleotide-binding thermodynamic signatures similar to each other, characterized by enthalpically-driven affinities in the μM range. Nevertheless, contrary to the catalytic subunit that recognizes MgATP and MgADP with comparable strength, the noncatalytic subunit much prefers the triphosphate nucleotide. Besides, the α-subunit depends more on Mg(II) for stabilizing the interaction with ATP, while both subunits are rather metal-independent for ADP recognition. These binding behaviors are discussed in terms of the properties that the two subunits exhibit in the whole enzyme.

1. Introduction

Rotary enzymes are among the most sophisticated molecular devices in nature. The ability of these enzymes to interconvert mechanical and chemical energies is used in processes such as ATP synthesis and ATP-driven ion transport across membranes [1]. F_1 subcomplex, the water-soluble sector of F_0F_1-ATP synthase that carries the catalytic machinery, is one of the most thoroughly-studied motor enzymes [2–5]. In its minimal architecture with full ATPase activity, F_1 is a heptamer composed of three different subunits: $\alpha_3\beta_3\gamma$. Each β-subunit has a catalytic site which is complemented with some residues of the adjacent α-subunit. Conversely, three noncatalytic sites are located at the α-/β-subunit interfaces. Although many aspects of F_1's rotary mechanism remain to be clarified, the available wealth of experimental and theoretical data has provided the basis for a deep understanding of the complex role played by catalytic subunits. Studies in the last decade based on single-molecule spectroscopy have been particularly insightful in unveiling the basic coupling scheme for hydrolysis-driven rotation [4,6,7]. In contrast, the molecular basis for nucleotide recognition and inter-subunit communication by noncatalytical subunits is much less understood.

α-Subunits have proven necessary for attaining maximum steady-state activity in F_1-ATPase and F_0F_1-ATP synthase from different species [8–10]. The presence of at least one active α-subunit is necessary to

Abbreviations: Mg(II), free magnesium ion; ATP, adenosine 5′-triphosphate; ADP, adenosine 5′-diphosphate; EDTA, ethylenediaminetetraacetic acid; Tris, 2-amino-2-(hydroxymethyl)propane-1,3-diol; MgCl2, magnesium chloride; ATPase, adenosine 5′-triphosphatase; TF1, F1 sector from *Bacillus* PS3; EF1, F1 sector from *Escherichia coli*; S1, S2 and S3, high, medium and low affinity β-subunit sites in F1, respectively; βDP and βTP, β subunits in the crystal structure of F1 bound to Mg·ADP and Mg·AMPPNP, respectively; ITC, isothermal titration calorimetry; NMR, nuclear magnetic resonance; K_d, equilibrium dissociation constant; K_b, equilibrium binding constant; ΔH_b, binding enthalpy; ΔG_b, binding Gibbs free energy; ΔS_b, binding entropy; K_{N1} and K_{N2}, step-wise association constants for the interaction of ATP with Mg(II); K_{Pf} and K_{Pb}, association constants for ATP and Mg·ATP binding to the subunit; κ, cooperative heterotropic association constant; Δg, cooperative Gibbs free energy; Δh, cooperative enthalpy; Δs, cooperative entropy

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release inhibitory MgADP entrapped at catalytic sites [11]. Recent studies based on high-speed atomic force microscopy have shown that catalytic sites in the $\alpha_3\beta_3$ subcomplex, i.e., in the absence of the γ-subunit, also exhibit ATP-induced dynamic conformational changes and binding asymmetry [12]. This observation provides compelling evidence that α-subunits do transmit conformational signals between β-subunits, confirming previous results [13–15]. It has been well-documented that isolated β-subunits undergo large conformational changes upon nucleotide binding, and that these closely resemble the transition between the open and closed conformations exhibited in F_1 [16]. In contrast, available information indicates that the α-subunit exhibits much more limited conformational changes upon ligand binding than those undergone by the β-subunit [17].

A deep understanding of the forces that drive conformational changes of α- and β-subunits, as well as the coordination and cooperativity among them, is crucial to unveil the molecular basis of F_1-ATPase function. To pursue that goal, the nucleotide-recognition energetics of F_1 needs to be known in detail. Nevertheless, the enzyme's complex mechanism, involving different modes of ligand and intercatenary interactions that take place in a coordinated and cooperative way among catalytic and noncatalytic subunits, has precluded unambiguous quantification of the underlying energetics [18]. To gain more insights into the forces governing F_1 function, we have calorimetrically characterized the nucleotide-recognition ability of the isolated α-subunit. Thermodynamic signatures for the interaction with metal-free and metal-bound nucleotides were obtained in order to assess the energetic role of Mg(II). Additionally, a comparison was performed with previously-reported binding properties of isolated β-subunit from thermophilic *Bacillus* PS3 [19,20]. Overall, the analysis reveals that the two isolated subunits exhibit very similar energetic strategies for recognizing nucleotides, in spite of the low identity of their polypeptide sequences. In turn, these properties correlate with binding behaviors exhibited by each subunit within F_1.

2. Materials and methods

2.1. Materials

All chemicals, including ATP and ADP sodium salts, were from Sigma Chemical Co. Plasmids pGAB1 encoding full length F_1 of ATPase were kindly provided by Hiroshi Omote (The Institute of Scientific and Industrial Research, Osaka University). α-Subunit of F_1 was amplified by PCR and subcloned into BamH1 and HindIII restricted pET28 vector (Novagen).

Early reports documented that isolated α-subunits from different sources are prone to aggregate in solution [21,22]. We observed a similar behavior with recombinant α-subunit from *Bacillus* PS3. The protein was stable as a monomer inasmuch as no stirring was applied. However, even mild stirring caused protein precipitation, a situation that precluded calorimetric measurements. Attempts to inhibit aggregation (variation of pH, ionic force, osmolyte content, temperature) were unsuccessful. In view of these results, we decided to express the α-subunit from *Geobacillus kaustophilus*, which differs from that of *Bacillus* PS3 by 12 residue substitutions, most of them occurring at the protein surface (Supplementary data). The *Geobacillus* subunit proved less prone to aggregation than the *Bacillus* subunit. In the presence of 10% glycerol, precipitation was minimal throughout an ITC experiment, as judged from visual inspections of the samples and from the resulting fitting binding stoichiometry (see footnote to Table 1). Therefore, the *Geobacillus* α-subunit was used to perform thermodynamic characterizations.

Table 1
Binding energetics of the α-subunit to free and Mg(II)-bound adenosine nucleotides[a,b].

Ligand	K_b[c] × 10^{-3} (M^{-1})	ΔG_b (kcal/mol)	ΔH_b (kcal/mol)	$-T\Delta S_b$ (kcal/mol)
MgADP	62 ± 16	−6.5	−6.4 ± 0.2	−0.1
MgATP	616 ± 13	−8.1	−12.1 ± 1.2	4.0
ADP	27 ± 2	−6.1	−4.6 ± 0.2	−1.5
ATP	1.8 ± 0.1	−4.4	−3.9 ± 0.4	−0.5

[a] 25 °C, in a 0.05 M Tris–HCl buffer solution supplemented with 0.1 M NaCl and 10% glycerol (pH 8.0).
[b] In the presence of Mg(II), both nucleotides yielded a fitting binding stoichiometry of 1.04 ± 0.01. In the absence of the metal ion, the stoichiometry was fixed to 1 in order to get convergence in the fitting process.
[c] K_b corresponds to K_{Pf} and K_{Pb} in Scheme 1 for the binding of Mg(II)-free or Mg(II)-bound nucleotide to the subunit, respectively.

2.2. Expression of recombinant Geobacillus α subunit

The recombinant α subunit was overexpressed in *Escherichia coli* BL21 cells (Novagen) harboring plasmid p28-α. LB broth supplemented with kanamycin (30 μg/ml) was used to grow cells at 37 °C. A 1:100 dilution for the overnight growth into fresh LB was made and allowed to grow until A600 reached 0.6. At this point, IPTG was added at 1 mM final concentration to induce protein expression. The cells were incubated for 4 h at 37 °C and harvested by centrifugation.

2.3. Protein purification

α-Subunit was purified to homogeneity as previously described [22]. In brief, harvested cells were lysed with lysis buffer (50 mM Tris–HCl, 250 mM NaCl, 5 mM imidazole, 10% glycerol, pH 8.0) by sonication at 4 °C in a Misonix 3000 sonicator. The soluble and insoluble fractions were separated by centrifugation (32,000 ×*g*/45 min/4 °C) and analyzed by SDS-PAGE. The over production of recombinant protein was found in the soluble fraction. Recombinant His6-tagged α subunit was purified by metal-chelate affinity chromatography using a HiTrap Ni^{2+} column (GE Healthcare), followed by a Hi/prep Mono Q column (Amersham Biosciences).

All experiments were performed at 25 °C, in 0.05 M Tris–HCl buffer solution, pH 8.0, supplemented with 0.1 M NaCl and 10% glycerol. Purified α-subunit was thoroughly dialyzed against the buffer solution prior to calorimetric measurements. Assays with metal-bound nucleotide were performed with ligand and protein solutions supplemented with 5 mM $MgCl_2$. For the titration with Mg(II)-free nucleotide, 2 mM EDTA was used to sequester any residual trace of the metal. After degasification, protein concentration was determined using the modified Bradford assay (Bio-Rad, Germany). For ADP and ATP, an extinction coefficient of 15,600 M^{-1} cm^{-1} at 259 nm was used. $MgCl_2$ and nucleotides were dissolved in the buffer solution obtained in the last dialysis. The solution's pH was re-adjusted to 8.0 using NaOH when necessary.

2.4. Isothermal titration calorimetry

Calorimetric measurements were carried out with a MicroCal™ iTC_{200} System (GE Healthcare, Northampton, MA, USA). α-Subunit concentration was typically 0.02–0.03 mM. The titration schedule consisted of 12–20 consecutive injections of 3 μL with a 5-min interval between injections, using a stirring rate of 700 rpm. The dilution heat of the ligand was obtained by adding ligand to a buffer solution under conditions and injection schedule identical to those used with the protein sample. In the case of the titration of the α-subunit with Mg(II)-free nucleotide, the binding constant (K_{P1}) and the enthalpy change (ΔH_{P1}) were determined by the nonlinear fitting of a single binding-site model [23]. The resulting binding isotherms in the presence of Mg(II) were analyzed using a ternary model in which the α-subunit (P) may bind AT(D)P or MgAT(D)P, while ATP may also be in the form of Mg_2ATP (see Scheme 1). Derivation of the coupled-equilibria model has been

$$\begin{array}{ccccccccc} & & & \overset{K_{N1},\Delta H_{N1}}{} & & & & \overset{K_{N2},\Delta H_{N2}}{} & \\ AT(D)P & + & 2Mg(II) & \leftrightarrow & Mg-AT(D)P & + & Mg(II) & \leftrightarrow & Mg_2-ATP \\ + & & & & + & & & & \\ P & & & & P & & & & \\ \updownarrow & \overset{K_{Pf},\Delta H_{Pf}}{} & & & \updownarrow & \overset{K_{Pb},\Delta H_{Pb}}{} & & & \\ P-AT(D)P & + & 2Mg(II) & \leftrightarrow & P-MgAT(D)P & + & Mg(II) & & \\ & & & \underset{K_M,\Delta H_M}{} & & & & & \end{array}$$

Scheme 1. Ternary binding equilibrium among isolated α- or β-subunit of F_1-ATPase (P), Mg(II) and AT(D)P. The ability of ATP to form a bidentate complex with Mg(II) is indicated.

presented in detail elsewhere [20]. In brief, when applied to the interaction with MgATP, the binding model acquires the form:

$$\begin{aligned} q_i = V_0\Bigg(&\left([P\cdot ATP]_i - [P\cdot ATP]_{i-1}\left(1-\frac{v}{V_0}\right)\right)\Delta H_{Pf} \\ &+\left([P\cdot MgATP]_i - [P\cdot MgATP]_{i-1}\left(1-\frac{v}{V_0}\right)\right)(\Delta H_{Pb}+\Delta H_{N1}) \\ &+\left([MgATP]_i - [MgATP]_{i-1}\left(1-\frac{v}{V_0}\right) - F_{MgATP}[ATP]_0\frac{v}{V_0}\right)\Delta H_{N1} \\ &+\left([Mg_2ATP]_i - [Mg_2ATP]_{i-1}\left(1-\frac{v}{V_0}\right) - F_{Mg_2ATP}[ATP]_0\frac{v}{V_0}\right)(\Delta H_{N1}+\Delta H_{N2})\Bigg) + q_{dil} \end{aligned} \quad (1)$$

where ΔH_{Pf} and ΔH_{Pb} are the enthalpy changes associated with the binding of ATP and MgATP to the α-subunit, respectively; ΔH_{N1} and ΔH_{N2} are the enthalpy changes associated with the binding of the first and second Mg(II) to ATP, respectively, and q_{dil} is a fitting term introduced to account for experimentally uncorrected dilution heat effects. The correction terms including F_{MgATP} and F_{Mg2ATP} (the fractions of ATP in the syringe with one and two magnesium atoms bound, respectively, that can be calculated from the total ATP and Mg(II) concentrations in the syringe and the ATP-Mg(II) binding constants) correspond to the MgATP and Mg_2ATP complexes introduced in the cell just by injection and not due to equilibrium balance. Finally, $[ATP]_0$ stands for the ATP concentration in the syringe, v for the injection volume, and V_0 for the cell volume.

The total concentration of each species in Eq. (1) is related to the concentrations of free molecular counterparts through the equilibrium constants:

$$\begin{aligned} [ATP]_T = {} & [ATP] + K_{N1}[ATP][Mg(II)] + K_{N1}K_{N2}[ATP][Mg(II)]^2 \\ & + K_{Pf}[P][ATP] + K_{Pb}K_{N1}[P][ATP][Mg(II)][Mg]_T = [Mg(II)] \\ & + K_{N1}[ATP][Mg(II)] + 2K_{N1}K_{N2}[ATP][Mg(II)]^2 \\ & + K_{Pb}K_{N1}[P][ATP][Mg(II)][P]_T = [P] + K_{Pf}[P][ATP] \\ & + K_{Pb}K_{N1}[P][ATP][Mg(II)]. \end{aligned} \quad (2)$$

Finally, a parameter n was included as a normalization factor for correcting errors in protein concentration related to experimental determination and/or the presence of a fraction of inactive protein:

$$[P]_T = n[P]_0 \quad (3)$$

where $[P]_0$ is the experimental concentration. K_{N1}, ΔH_{N1}, K_{N2}, ΔH_{N2}, K_{Pf}, and ΔH_{Pf} are determined directly from direct binary titrations. K_{Pb} and ΔH_{Pb} are determined from the ternary experiments, once the other parameters have been previously determined. For the case of ADP, the model is adapted from the more general ATP model by simply setting $K_{N2} = \Delta H_{N2} = 0$.

The cooperativity in the binding of nucleotide and Mg(II) to the α-subunit is contained in K_{P2} and ΔH_{P2} relative to K_{P1} and ΔH_{P1} values [24,25]:

$$\kappa = K_{P2}/K_{P1} = K_M/K_{N1}, \quad (4)$$

$$\Delta h = \Delta H_{P2} - \Delta H_{P1} = \Delta H_M - \Delta H_{N1} \quad (5)$$

where K_M and ΔH_M stand for the association parameters of Mg(II) to the nucleotide-prebound α-subunit. Finally, the cooperative entropy change can be calculated as:

$$T\Delta s = \Delta h - \Delta g = \Delta h + RT\ln\kappa. \quad (6)$$

3. Results

In a previous study with the β-subunit from thermophilic *Bacillus* PS3, it was shown that, in order to describe correctly the interaction of the protein with nucleotides, it was necessary to consider explicitly the coupled binding equilibria depicted in Scheme 1 [20].

In the above scheme, subscripts N*i* stand for the association of Mg(II) to the nucleotide, Pf and Pb for the association of metal-free and metal-bound nucleotide to the protein, respectively, and M for the association of Mg(II) to the nucleotide-prebound protein. In this coupled equilibrium of weak interactions, AT(D)P and MgAT(D)P compete with each other for the subunit binding site (P). At the same time, mono and – in the case of ATP – bidentate interactions between the metal ion and the nucleotide take place. Consideration of these coupled equilibria was pertinent in the case of the β-subunit, as the affinities for metal-free nucleotides are not negligible and comparable in relation to those for the metal-bound nucleotides, while the affinities of the two nucleotides for Mg(II) are relatively weak [20].

Fig. 1 shows calorimetric isotherms obtained for the association of the α-subunit with MgAT(D)P or AT(D)P. It is evident that the presence of Mg(II) modifies the subunit's interaction with nucleotides, although the effect is sharper with ATP. In the case of metal-free nucleotides, a simple 1:1 binding equilibrium is established. Accordingly, the data were fitted using a single binding site model. In the presence of Mg(II), a binding model that takes into account the formation of all species in Scheme 1 was fitted to the calorimetric data. To reduce the number of degrees of freedom, experimental data for the α-subunit association with AT(D)P, as well as previously determined data for the formation of the complex MgADP or MgATP/Mg_2ATP [20], were used as fixed values.

Table 1 summarizes results obtained from analysis of the calorimetric data. Due to a binding enthalpy that is twice as favorable, the α-subunit recognizes MgATP with more affinity than MgADP. These differences are at variance with those in the β-subunit, which shows nearly the same thermodynamic signature for the recognition of the two metal-bound nucleotides (Fig. 2). The two subunits interact with MgADP with very similar strength ($K_{Pb} = 6.9 \times 10^4\ M^{-1}$ for β-subunit [20]), although with distinctly different enthalpic and entropic contributions. In contrast, the α-subunit binds to MgATP with more affinity (~6-fold) than the β-subunit ($K_{Pb} = 1.0 \times 10^5\ M^{-1}$ [20]).

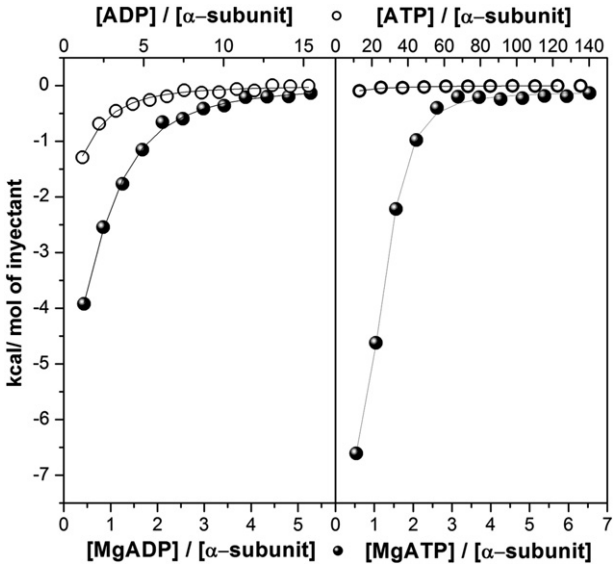

Fig. 1. Calorimetric isotherms for the interaction of isolated α-subunit with ADP (left panel) and ATP (right panel), at 25 °C, in a 0.05 M Tris–HCl buffer solution supplemented with 0.1 M NaCl and 10% glycerol (pH 8.0). Measurements were carried out in the presence (solid symbols) or absence (open symbols) of Mg(II).

In earlier studies, the affinity constants of isolated α-subunit for nucleotides were determined, but as far as we know, no other characterizations of the binding enthalpies and entropies have been carried out. As shown in Table 2, the spectroscopic values reported by Otha et al. [26] for the α-subunit from *Bacillus* PS3 are in fair agreement with the calorimetric values obtained in the present study, except for the recognition of Mg(II)-free ATP, for which we obtained a significantly larger dissociation constant (K_d). In contrast, Miyauchi et al. [27] using UV-difference spectroscopy, found virtually no affinity differences for the two nucleotides by *Bacillus* PS3 α-subunit. Table 2 shows somewhat different values with the isolated α-subunit from *E. coli*, which were determined through fluorimetric assays [22]. Also in this case, the data are consistent with the α-subunits presenting preference for MgATP, although a similar effect for the two nucleotides by Mg(II) was observed.

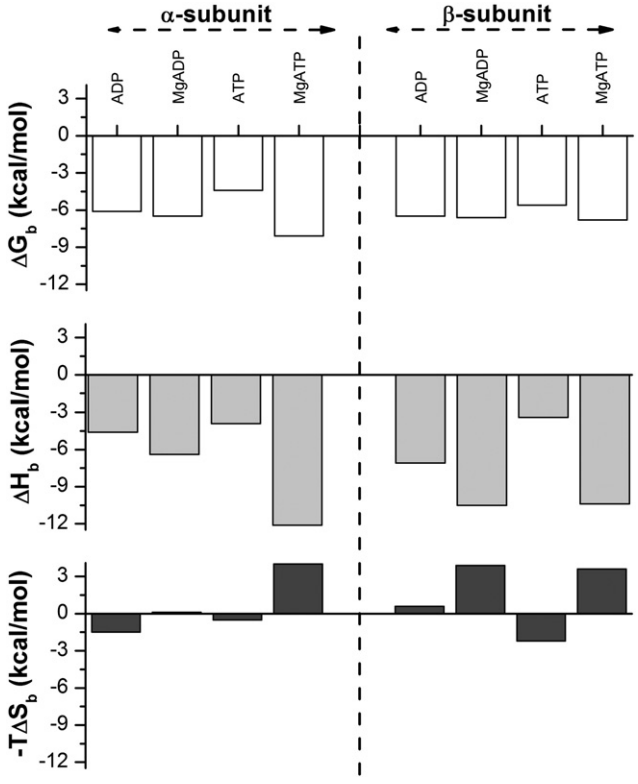

Fig. 2. Thermodynamic nucleotide-binding signatures of isolated α- and β-subunits. Values for the catalytic subunit were taken from [20].

Table 2
Comparison of nucleotide dissociation constants (K_d) of isolated α-subunit.

Nucleotide	Gα[a]	K_d (μM) Tα[b]	Tα[c]	Eα[d]
MgADP	16	18	11	4.4
MgATP	1.6	10	9	0.3
ADP	36	120	ND[e]	14.1
ATP	567	18	ND[e]	1.2

[a] This work.
[b] *Bacillus* PS3 subunit. Values taken from Otha et al. [26].
[c] *Bacillus* PS3 subunit. Values taken from Myauchi et al. [27].
[d] *E. coli* subunit. Values taken from Senior et al. [22].
[e] Not determined.

One approach to quantifying the energetic role of Mg(II) in the interaction is to calculate cooperativity parameters for the heterotropic interaction between Mg(II) and nucleotides. From a formal point of view, considering ATP as the reference binding molecule, this system represents an example of coupling between a cooperative homotropic interaction [ATP may bind one or two Mg(II) ions] and cooperative heterotropic interaction [ATP may bind the α-subunit, may bind Mg(II), or may bind both the α-subunit and Mg(II) simultaneously]. The cooperativity association constant, κ, enthalpy, Δh, entropy, Δs, and Gibbs free energy, Δg, can be calculated from thermodynamic parameters for the binding of the nucleotide and Mg-bound nucleotide to the α-subunit, and they reflect the reciprocal effect of each ligand on the binding to the subunit. κ values >1, <1 or = 1 indicate positive, negative or null cooperativity, respectively.

According to the data in Table 3, the affinity of the α-subunit for ADP is barely affected by Mg(II). In contrast, the affinity for ATP increases considerably in the presence of the metal ion due to a more favorable Δh value. A similar behavior is observed for the recognition of the two nucleotides by the β-subunit. Nevertheless, the more negative cooperativity entropy observed with the β-subunit makes the corresponding κ for ATP binding significantly smaller than that observed with the α-subunit.

An alternative way to evaluate the energetic effects of Mg(II) is by determining the "pure" Mg(II) binding contribution, i.e., the binding of the cation to a preformed subunit-nucleotide adduct:

$$P \cdot AT(D)P + Mg(II) \rightleftharpoons P \cdot AT(D)P{-}Mg \equiv P{-}Mg \cdot ATP,$$

which can be derived by applying Hess' law on the involved elementary binding equilibria and explicitly expressed in Eqs. (4) and (5).

Table 3
Cooperativity binding for the heterotropic interaction of Mg(II) and nucleotides with isolated α- and β-subunits at 25 °C.

	κ	Δg (kcal/mol)	Δh (kcal/mol)	−TΔs (kcal/mol)
α-MgADP	2	−0.5	−1.8	−1.3
α-MgATP	308	−3.4	−7.6	3.4
β-MgADP	1	−0.1	−3.4	3.3
β-MgATP	8	−1.2	−7.0	5.8

$$\begin{aligned} P + Mg{\cdot}AT(D)P &\rightleftharpoons P{-}Mg{\cdot}AT(D)P \\ P{-}AT(D)P &\rightleftharpoons P + AT(D)P \\ Mg(II) + AT(D)P &\rightleftharpoons Mg{-}AT(D)P \\ \hline P{\cdot}AT(D)P + Mg(II) &\rightleftharpoons P{-}Mg{\cdot}AT(D)P \end{aligned}$$

Scheme 2.

Binding parameters obtained through Scheme 2 contain information on the equilibrium between the free cation and nucleotides, therefore yielding contributions that differ quantitatively from heterotropic cooperativity parameters. Inspection of the data in Table 4 reveals that the interaction of Mg(II) with the two F_1 subunits is optimal in the presence of ATP. The γ-phosphate makes metal–ion binding both enthalpically and entropically driven. In contrast, binding enthalpy is unfavorable in the presence of ADP. Overall, similar to the heterotropic values, ΔG_M values indicate that the α-subunit's recognition site offers, in the presence of ATP, the best environment for Mg(II) anchoring.

4. Discussion

In this study, we have characterized calorimetrically the thermodynamic signatures for the association of the isolated F_1-ATPase α-subunit with adenosine nucleotides. Remarkably, the binding behavior of this subunit parallels to a large extent that shown by the isolated β-subunit. The two subunits exhibit enthalpically-driven affinities in the μM range for MgADP and MgATP. They show preference for binding to the three-phosphate nucleotide, although this preference is stronger in the case of the noncatalytic subunit. Another similarity is the nucleotide-specific effect that Mg(II) exerts on them. The cation yields positive heterotropic binding cooperativity in both subunits only in the presence of ATP, while its contribution with ADP is marginal. In both subunits, the metal-induced stabilizing effect is the result of a large favorable cooperative enthalpy. NMR and thermodynamic studies have shown that the isolated catalytic subunit adopts a more closed conformation when it is bound to MgATP than to MgADP or to metal-free nucleotides [16,20,28]. The more closed conformation has been correlated with the catalytically activated state that the β-subunit adopts transiently within F_1 [16,28], which in turn is more compact than any of the closed structures seen in the F_1 crystal structures so far reported. Analysis of the thermodynamic signatures reveals a similar conformational behavior for the noncatalytic subunits. Desolvation of the γ-phosphate group is expected to add extra favorable binding entropy. Yet, MgATP binding by the α-subunit is accompanied by more unfavorable entropy, while MgADP binding is isoentropic. It follows that, like the β-subunit, the α-subunit also adopts a more rigid conformation bound to MgATP.

Besides stereochemical considerations, the absence of Mg(II)-dependent cooperative effects with ADP seems to be due to the functional requirements of the enzyme, in particular to those related to the activity of catalytic sites. Walker and coworkers have recently presented structural evidence for a new intermediate step during ATP hydrolysis. In the structure of bovine F_1-ATPase cocrystallized with ADP and low concentrations of Mg(II), the catalytic site of the β-subunit, in the extended conformation (β_E), was occupied by metal-free ADP [29]. The same interaction was captured in the crystal structure of yeast F_1-ATPase bound to the inhibitory protein IF_1 [30]. According to the authors, these structures unveil a sequential product release mechanism, with the ADP molecule being liberated after release of the phosphate and magnesium ions. Single-molecule observations have revealed that ATP is hydrolyzed at a rotation angle of ~200°, while ADP is released at ~240°. Accordingly, Mg(II) and phosphate should be released somewhere between ~200° and ~240° [31]. Our calorimetric study provides the energetic basis for this sequential mechanism, given that the interaction strength of the β-subunit with ADP is unaffected by Mg(II), thus expediting cation pre-liberation. Whether this absence of cooperative effects has any implication for the function of α-subunits is a question that needs to be elucidated.

A striking difference between the two isolated subunits is the larger affinity that the α-subunit exhibits toward MgATP. As shown in Table 3, Mg(II) is largely responsible for this increased affinity, as judged from the 40-fold larger κ value of the α-subunit in relation to that of the β-subunit. Interestingly, it is only in the presence of Mg(II) that noncatalytic subunits bind tightly to ATP and release inhibitory ADP at catalytic sites [32]. This behavior is in qualitative agreement with the strong cation-dependence of the isolated α-subunit for recognizing ATP.

Fig. 3 shows schematic representations of the binding sites of α_{TP}- and β_{TP}-subunits occupied by AMP-PNP, as observed in the crystal structure of bovine F_1 in the so-called "ground state" conformation [33]. It can be seen that identical interactions are established by the metal ion in the two subunits. Thus, the distinct stabilization effect that Mg(II) elicits must be indirect. In this regard, it is noteworthy that the ATP γ-phosphate group forms interactions with different residues in the two subunits. While in the α-subunit it is stabilized by the main-chain and side-chain amides of Q^{172}, in the β-subunit it interacts with the side-chain polar group of R^{189} and R^{373}. Thus, it is tempting to propose that the more unfavorable heterotropic entropy effect that Mg(II) brings about on the β-subunit in the presence of ATP (Table 3) is because a larger number of the protein's rotatable bonds freeze upon binding. However, further studies are required to unveil the molecular basis of the differences in binding behaviors exhibited by the α- and β-subunits, since other effects may be operating in a complex way. For instance, the β-phosphate group interacts with P-loop residues and with Mg(II) in an identical way in the two subunits. As stated in Section 1, Introduction section, the β-subunit undergoes large conformational changes upon ligand binding, while the α-subunit seems to prefer a closed conformation, regardless of its ligation state. Hence, a less favorable binding entropy of β-subunit with MgADP would be expected. However, our calorimetric data revealed the opposite behavior.

Several studies have dealt with the nucleotide-binding strength of noncatalytic sites in F_1. However, determination of binding affinities in F_1 has historically been a very complicated task, and most available data can be considered as apparent constants [18]. One of the many factors that complicate measurements is that noncatalytic subunits of F_1 from different species exchange nucleotides slowly, although this regime is preceded by a fast and reversible association. Weber et al. [34], based on fluorimetric measurements, obtained equilibrium constants for the rapid association event in F_1 from *E. coli*, which indicated that noncatalytic subunits bind more tightly to MgATP (K_d = 55 μM) than to MgADP (K_d = 105 μM). The authors also reported dissociation constants for magnesium-free nucleotides (K_d = 3.5 and 1.3 mM for free ATP and ADP, respectively). Using data presented in that study in Eqs. (4) to (6) (Section 2, Materials and methods), the heterotropic cooperative constant of Mg(II) and ATP for binding to a noncatalytic subunit in F_1 is 64, while the corresponding κ value for ADP is 13. Thus, it would seem that regardless of their quaternary state, α-subunits show the same relative nucleotide preference as well as

Table 4
Thermodynamic parameters for the binding of Mg(II) to the subcomplex:[a].

Subcomplex	ΔG_M (kcal/mol)	ΔH_M (kcal/mol)	$-T\Delta S_M$ (kcal/mol)
α-ADP	−5.0	1.9	−6.9
α-ATP	−9.9	−3.8	−5.1
β-ADP	−4.7	0.3	−5.0
β-ATP	−7.4	−2.6	−4.8

[a] Values were calculated on the basis of Scheme 2. Experimental conditions: T = 25 °C, pH 8.0, 0.05 M Tris–HCl, 0.1 M NaCl.

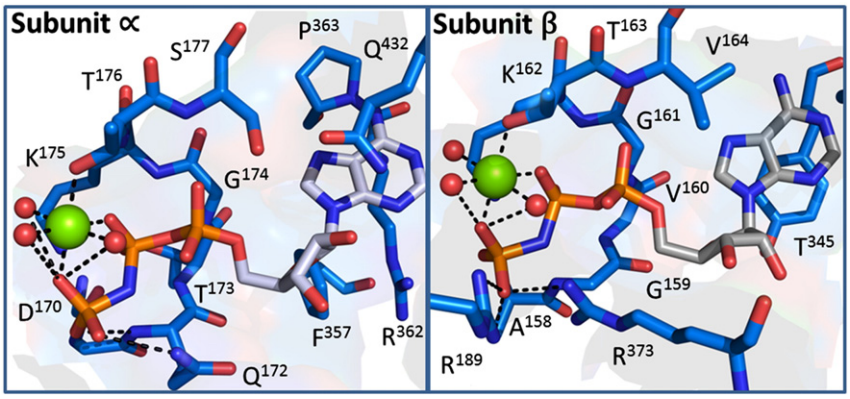

Fig. 3. Schematic representation of the binding sites of subunits α (left) and β (right) of F_1-ATPase. Coordinates for the TP conformations of both subunits bound to AMP-PNP were extracted from the pdb file 2JDI [32], corresponding to the crystal structure of azide-free bovine F_1-ATPase ("ground state" conformation).

stabilizing effect by Mg(II). However, the binding energetics by noncatalytic subunits in F_1 is far from being thoroughly clarified.

Overall, our study sheds new light on the energetic basis of nucleotide recognition by F_1-ATPase subunits. In spite of genetic variation and functional divergence, α- and β-subunits in monomeric state have preserved similar energetic strategies for binding to nucleotides. This is not completely surprising, since the two subunits share similar binding-site architectures (Fig. 3). In particular, the interaction of the P-loop with the nucleotide is virtually identical in the two subunits. Our data reveal that Mg(II) has a substantial effect on shaping the nucleotide-recognition sites, allowing an optimal and more rigid packing in the presence of ATP. At the same time, the two subunits show quantitative differences in binding parameters that correlate with the roles they play in F_1. Nevertheless, to understand how the oligomeric environment modulates binding behaviors of the subunits and determines communication among them, a study aimed at unveiling the energetics of the complex F_1's binding mechanism is required.

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