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Empirical Estimation of Interaction Energies for Ligands Binding in the Isolated β-Subunit of F_0F_1 ATP Synthase from *Rhodospirilum rubrum*†

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ABSTRACT: Under standard experimental conditions one site of the isolated β-subunit of F_0F_1 from *Rhodospirilum rubrum* binds ATP or ADP with $\Delta G°_{ATP}(\beta_1) = -7.6$ kcal/mol and $\Delta G°_{ADP}(\beta_1) = -7.4$ kcal/mol, while the second site binds ATP, ADP, or P_i with $\Delta G°_{ATP}(\beta_2) = -5.1$ kcal/mol, $\Delta G°_{ADP}(\beta_2) = -5.6$ kcal/mol, and $\Delta G°_{P}(\beta_2) = -4.9$ kcal/mol. The synthesis–hydrolysis of ATP on the second site of β can be described by

$$ADP + P_i + E \overset{\Delta G°_{DP}}{\rightleftharpoons} E{\cdot}ADP{\cdot}P_i \overset{\Delta G°_{B}}{\rightleftharpoons} E{\cdot}ATP \overset{\Delta G°_{ATP}}{\rightleftharpoons} E + ATP \overset{\Delta G°_{N}}{\rightleftharpoons} E + ADP + P_i \quad (1)$$

in which $\Delta G°_{DP} = -7.1$ kcal/mol, $\Delta G°_{B} = +9.6$ kcal/mol, $\Delta G°_{ATP} = +5.1$ kcal/mol, and $\Delta G°_{N} = -7.6$ kcal/mol. This suggests that the binding energy of both ATP ($\Delta G°_{ATP}$) and ADP + P_i ($\Delta G°_{DP}$) is weak, causing a very unfavorable enzyme-bound ATP synthesis, $\Delta G°_{B} = +9.6$ kcal/mol. This value of $\Delta G°_{B}$ is very different from the value of $\Delta G°_{B} = -0.4$ kcal/mol observed in the single catalytic site of F_1. This large difference in $\Delta G°_{B}$ values ($\Delta\Delta G°_{B} = +10$ kcal/cal) is caused by the difference in ATP binding ($\Delta\Delta G°_{ATP} = +11.2$ kcal/mol). The overall binding energy of ADP + P_i is not so different in two experimental systems, $\Delta\Delta G°_{DP} = +1.2$ kcal/mol, and it cannot account for such a large difference in $\Delta G°_{B}$ values. It is postulated that the strong binding site for ATP in F_1 is formed by two weak "half-sites", located on the different and neighboring copies of β-subunit. The ATP binding to two half-sites with $\Delta G°_{ATP}(\beta_1) = -7.6$ kcal/mol and $\Delta G°_{ATP}(\beta_2) = -5.1$ kcal/mol could give a strong binding to F_1 with $\Delta G°_{ATP}(F_1) = -16.3$ kcal/mol if we assume that the increase in binding energy caused by a two-step binding to two half-sites is $\Delta G^{S}(\beta_{12}) = (-7.6) + (-5.1) - (-16.3) = +3.6$ kcal/mol. This estimated value of $\Delta G^{S}(\beta_{12})$ is reasonably close to the observed value of $\Delta G^{S}(F_1) = +2.9$ kcal/mol in F_1, supporting the model in which a catalytic unit in F_1 is formed between two β-subunits.

The membrane-bound H^+-ATPase (F_0F_1 complex) of energy-transducing membranes (bacteria, chloroplasts, and mitochondria) is able to use an electrochemical gradient of protons ($\Delta\mu H^+$) for steady-state ATP synthesis (Mitchell, 1966; Racker, 1977; Kagawa et al., 1978; Kagawa, 1984). The H^+-ATPase has two main portions, a catalytic component F_1 composed of five subunits (α, β, γ, δ, ϵ) with a stoichiometry

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$\alpha_3\beta_3\gamma_1\delta_1\epsilon_1$ and a membrane-bound H^+-conducting F_0 portion composed of at least three subunits (a, b, c) with a possible stoichiometry $a_1b_2c_{8-10}$ (Senior & Wise, 1983; Amzel & Pedersen, 1983). The F_1 portion contains six nucleotide binding sites (three catalytic and three noncatalytic), which are restricted to the largest subunits α and β (Cross & Nalin, 1982; Boulay et al., 1985; Kironde & Cross, 1987). Three catalytic sites of F_1 show a strong negative cooperativity during the multiple turnover (Grubmeyer et al., 1982; Cross et al., 1982; O'Neal & Boyer, 1984; Melese & Boyer, 1985; Wang, 1985). The catalytic β-subunit shows 65–70% homology from bacteria to mitochondria (Futai & Kanazawa, 1983; Walker et al., 1984), which is clearly reflected in the function of the enzyme (Futai & Kanazawa, 1983; Gromet-Elhanan et al., 1985; Richter et al., 1986). The catalytic β-subunit shows also structural similarities with other enzymes (Walker et al., 1984; Fry et al., 1986; Duncan et al., 1986; Garboczi et al., 1988).

The isolated and reconstitutively active α- and β-subunits are very useful experimental systems for the study of ligand binding. The main advantage for such an isolated system is that there is no significant catalytic turnover in the absence of subunit–subunit interaction (Dunn & Futai, 1980; Ohta et al., 1980; Gromet-Elhanan & Khananshvili, 1984; Issartel & Vignais, 1984). The isolated α-subunit contains one nucleotide binding site (Ohta et al., 1980; Dunn & Futai, 1980), while the isolated β-subunit has two nonidentical nucleotide binding sites (Gromet-Elhanan & Khananshvili, 1984; Khananshvili & Gromet-Elhanan, 1984, 1985a,b, 1986). One site of the isolated β-subunit binds either ATP or ADP in a Mg^{2+}-independent manner (designated the Mg^{2+}-independent site). The second site binds ATP, ADP, or P_i only in the presence of Mg^{2+} (designated the Mg^{2+}-dependent site). These binding sites probably exist also in the assembled F_1 enzyme, but we still do not know how they are oriented in the multisubunit F_1 enzyme (Bullough & Allison, 1986; Kironde & Cross, 1987; Cross et al., 1987; Joshi & Wang, 1987; Xue et al., 1987; Garboczi et al., 1988).

The mechanism of F_0F_1 is still unknown, and a number of proposals have been suggested (Mitchell, 1966, 1974; Boyer 1974, 1984; Boyer et al., 1978; Jencks, 1980, 1983). Under Uni-site catalytic conditions (when only one catalytic site operates in F_1) the K_{eq} for enzyme-bound ATP synthesis-hydrolysis is shifted toward ATP synthesis (Grubmeyer et al., 1982; Cross et al., 1982; O'Neal & Boyer, 1984). Although a $\Delta\mu H^+$-dependent release of strongly bound ATP from the single catalytic site of the membrane-bound enzyme was demonstrated (Penefsky, 1985a,b), it still is not clear how this process can take place without reversion of ATP back to ADP and P_i.

One way to characterize the binding energies in the enzymatic systems is to use "linked functions" or "interaction energies" (Wyman, 1964; Weber, 1975; Hill, 1977; Jencks, 1975, 1980, 1981; Ackers et al., 1983). The question is how "intrinsic binding energies" of specific moieties of ligands can be utilized in order to reverse the enzyme-bound ATP synthesis–hydrolysis. In this context it is relevant to ask: Is the isolated β-subunit able to use the intrinsic binding energies of specific moieties in order to drive the enzyme-bound ATP synthesis? If not, what makes possible the utilization of binding energies in the single catalytic site of F_1? In this paper an empirical approach (Jencks, 1981) was used to characterize interaction energies for different ligands binding in the isolated β-subunit.

Materials and Methods

The β-subunit of RrF_1[1] was extracted from chromatophores of *Rhodospirilum rubrum* by LiCl treatment and purified to homogeneity by chromatography on DEAE-Sephadex and DE-23 as outlined before (Khananshvili & Gromet-Elhanan, 1982; Gromet-Elhanan & Khananshvili, 1986). Different preparations of purified β-subunit restore 70–95% of both phosphorylation and ATPase activities in β-less RrF_0F_1 chromatophores with a specific activity of reconstitution of about 50–60 units/mg of protein. Purified preparations of β were stored in liquid nitrogen. Preparations used in this work showed a single band on gels when about 200 μg of purified β-subunit was used for SDS–PAGE according to the method of Weber and Osborn (1969) or Laemmli (1970). No Mg^{2+} or Ca^{2+} ATPase activities have been detected for the purified preparations of β-subunit, measured by using [γ-^{32}P]ATP (the sensitivity for the measurement of ATPase activity was 0.1–0.3 nmol of released P_i (mg of β)$^{-1}$ h^{-1}. Purified preparations of β show minor exchange activities: ATP ⇌ P_i [1–3 nmol (mg of β)$^{-1}$ h^{-1}] and ADP ⇌ P_i [0.5–2.0 nmol (mg of β)$^{-1}$ h^{-1}. It is possible that ATP ⇌ P_i exchange includes combined reactions, catalyzed by adenylate kinase (ATP ⇌ ADP exchange) and polynucleotide phosphorylase (ADP ⇌ P_i exchange). Very similar exchange reactions were observed in the partially purified preparations of F_0F_1 from *R. rubrum* (Oren et al., 1980). ATP ⇌ P_i exchange was nearly completely inhibited by AP_5A in the purified preparations of β. This suggests that AP_5A inhibits the ATP ⇌ ADP exchange and prevents formation of ADP, which is a substrate for the ADP ⇌ P_i exchange.

Before any interaction of β with ligand, the storage buffer was removed and ATP-free preparations of β were obtained as described before (Khananshvili & Gromet-Elhanan, 1985a,b). ATP-depleted preparations of β can be stored in 20% glycerol in liquid nitrogen for at least two weeks without any loss of activity. Ligand binding to β was measured by incubating ^{3}H- and ^{32}P-labeled ligand with β under experimental conditions described before (Gromet-Elhanan & Khananshvili, 1984; Khananshvili & Gromet-Elhanan, 1984, 1985a,b). Binding was initiated by addition of β (10 μM final concentration) to the buffer containing 50 mM Tricine–NaOH, pH 8.0, 5 mM $MgCl_2$, and 50 mM NaCl. After incubation for 1 h at 25 °C, unbound ligand was removed by elution–centrifugation (Penefsky, 1977; Khananshvili & Gromet-Elhanan, 1985a,b). Samples of 70–100 μL were placed in Eppendorf yellow tips (200 μL) that were inserted about 3–5 mm above the top of 1-mL Sephadex G-50 columns precooled at 4 °C (a sample cannot enter the column until centrifugation is initiated). The effluent from each column was diluted with 1 mL of water, and aliquots were assayed for radioactivity and protein content. The recovery of protein in the column effluent was above 90% when samples contained more than 0.5 mg of protein/mL. In control experiments (in the absence of β) less than 0.0005% of the applied radioactivity appeared in the effluent. Ligand-binding data were calculated by using a molecular weight of 50 000 for the β-subunit of RrF_1 (Bengis-Garber & Gromet-Elhanan, 1979).

Nonradioactive ATP and ADP were purified on Dowex AG 1-X4 by elution with 60 mM HCl (ADP) and 1 M HCl (ATP) and adjusted to pH 7.0 by Tris. Collected fractions were then lyophilized and desalted by passing through Sephadex G-10. [2,8-^{3}H]ATP (23–29 Ci/mmol) and [2,8-^{3}H]ADP (25–30

[1] Abbreviations: RrF_0F_1, F_0F_1 enzyme of *R. rubrum*; EDTA, ethylenediaminetetraacetic acid; SDS–PAGE, polyacrylamide gel electrophoresis with sodium dodecyl sulfate; DE-23, (diethylaminoethyl)cellulose; DEAE-Sephadex, (diethylaminoethyl)-Sephadex; Tricine, *N*-[tris(hydroxymethyl)methyl]glycine; Tris, tris(hydroxymethyl)aminomethane; AP_5A, P^1,P^5-bis(5′-adenosyl) pentaphosphate.

Table I: Comparison of K_{ATP}, K_{ADP}, and K_P Values between the Isolated β-Subunit and the Single, High-Affinity Catalytic Site of F_1

association constants for ligand binding to the isolated β-subunit[a]		association constants for ligand binding to the F_1 enzyme,[b] high-affinity catalytic site (M^{-1})
Mg-independent (M^{-1})	Mg-dependent (M^{-1})	
$K^{ATP}(\beta_1) = 2.0 \times 10^5$	$K^{ATP}(\beta_2) = 5.0 \times 10^3$	$K^{ATP}(F_1) = 1.0 \times 10^{12}$
$K^{ADP}(\beta_1) = 1.4 \times 10^5$	$K^{ADP}(\beta_2) = 1.1 \times 10^4$	$K^{ADP}(F_1) = 3.3 \times 10^6$
ND[c]	$K^{P}(\beta_2) = 3.7 \times 10^3$	$K^{P}(F_1) = 1.6 \times 10^3$
Ratios of Associated Constants		
$K^{ATP}(F_1)/K^{ADP}(F_1) = 3 \times 10^5$		$K^{ATP}(F_1)/K^{ATP}(\beta_1) = 5 \times 10^6$
$K^{ATP}(\beta_1)/K^{ADP}(\beta_1) = 1.5$		$K^{ATP}(F_1)/K^{ATP}(\beta_2) = 2 \times 10^8$
$K^{ATP}(\beta_2)/K^{ADP}(\beta_2) = 0.5$		$K^{ADP}(F_1)/K^{ADP}(\beta_1) = 25$
		$K^{ADP}(F_1)/K^{ADP}(\beta_2) = 3.0 \times 10^2$
		$K^{P}(F_1)/K^{P}(\beta_2) = 2.2$

[a] The binding of ATP, ADP, or P_i to the isolated β-subunit was measured under standard experimental conditions (50 mM Tricine–NaOH, pH 8.0, 50 mM NaCl at 25 °C) in the presence or absence of 5 mM $MgCl_2$ (see Materials and Methods). In the absence of Mg^{2+} and saturating concentrations of ligand a maximal stoichiometry of binding was 0.95 mol of ATP/mol of β, 0.97 mol of ADP/mol of β. In the presence of Mg^{2+} a maximal stoichiometry of 1.94 mol of ATP/mol of β, 1.86 mol of ADP/mol of β, and 0.89 mol of P_i/mol of β was obtained with saturating concentrations of ligand. Mg-dependent binding for ATP and ADP was calculated by subtraction of Mg-independent binding from the total binding in the presence of Mg^{2+} at given concentration of nucleotide (Mg^{2+} does not change the binding affinity of ADP and ATP to the Mg-independent site; Gromet-Elhanan & Khananshvili, 1984; Khananshvili & Gromet-Elhanan, 1985b). [b] K values were taken from the following references: Kasahara and Penefsky (1978), Cross et al. (1982), and Grubmeyer et al. (1982). [c] Not detected.

Table II: Interaction Energies for Ligands Binding in the Isolated β-Subunit and F_1[a]

$\Delta G°_{ATP}(\beta_2) = -5.1$[b]	$\Delta G°_{ATP}(F_1) = -16.3$[b]	$\Delta\Delta G°_{ATP} = +11.2$
$\Delta G°_{ADP}(\beta_2) = -5.6$[b]	$\Delta G°_{ADP}(F_1) = -9.0$[b]	$\Delta\Delta G°_{ADP} = +3.4$
$\Delta G°_{P}(\beta_2) = -4.9$[b]	$\Delta G°_{P}(F_1) = -4.3$[b]	$\Delta\Delta G°_{P} = -0.6$
$\Delta G^{i}_{ATP}(\beta_2) = +0.3$	$\Delta G^{i}_{ATP}(F_1) = -19.2$	$\Delta\Delta G^{i}_{ATP} = +19.5$
$\Delta G^{i}_{ADP}(\beta_2) = -0.2$	$\Delta G^{i}_{ADP}(F_1) = -11.9$	$\Delta\Delta G^{i}_{ADP} = +11.7$
$\Delta G^{i}_{P}(\beta_2) = +0.5$	$\Delta G^{i}_{P}(F_1) = -7.3$	$\Delta\Delta G^{i}_{P} = +7.8$
$\Delta G°_{DP}(\beta_2) = -7.1$	$\Delta G°_{DP}(F_1) = -8.3$	$\Delta\Delta G°_{DP} = +1.2$
$\Delta G^{S}(\beta_2) = -5.4$[b]	$\Delta G^{S}(F_1) = +2.9$	$\Delta\Delta G^{S} = -8.3$
$\Delta G_{12}(\beta_2) = +3.4$[b]	$\Delta G_{12}(F_1) = +5.1$	$\Delta\Delta G_{12} = -1.7$
$\Delta G_{I}(\beta_2) = -2.0$	$\Delta G_{I}(F_1) = +8.0$	$\Delta\Delta G_{I} = -10.0$
$\Delta G°_{B}(\beta_2) = +9.6$	$\Delta G°_{B}(F_1) = -0.4$[b]	$\Delta\Delta G°_{B} = +10.0$

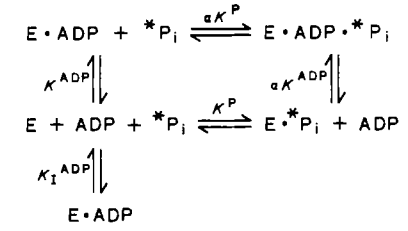
[a] All units in kcal/mol (standard state for 1 M). [b] Values were directly measured in the experiment for the Mg-dependent binding site of β in this work (for details see text) or for F_1 (Cross et al., 1982; Grubmeyer et al., 1982; Kasahara & Penefsky, 1977). All other values were estimated from the available experimental data as described in the text.

Ci/mmol) were obtained from New England Nuclear (>97% purity). Before usage of radioactive nucleotides, ethanol was evaporated from the solution under a stream of nitrogen or argon, and they were dissolved in buffer solution. [^{32}P]P_i was obtained from the Nuclear Research Center, Negev, Israel, and purified on Dowex AG 1-X4 as described before (Khananshvili & Gromet-Elhanan, 1985a,b); it contained <0.0001% impurities as estimated by an isobutyl alcohol–benzene extraction procedure (Avron, 1960).

Results

Estimation of ΔG^{i}_{ATP}, ΔG^{i}_{ADP}, *and* ΔG^{i}_{P}. The binding of ATP, ADP, or P_i to the isolated β-subunit depends on the pH and Mg^{2+} (Gromet-Elhanan & Khananshvili, 1984; Khananshvili & Gromet-Elhanan 1984, 1985,a,b). For example, the maximal binding stoichiometry of P_i occurs with low pH and Mg^{2+} concentrations (pH 6.5 and 1 mM $MgCl_2$) or with high pH and Mg^{2+} concentrations (pH 8.0 and 5 mM $MgCl_2$). In this work standard experimental conditions were chosen: 50 mM Tricine–NaOH pH 8.0, 50 mM NaCl, and 5 mM $MgCl_2$ at 25 °C, which are optimal for the binding of all three ligands, ATP, ADP, or P_i. In the absence of Mg^{2+} 1 mol of ATP or ADP binds per mole of the isolated β-subunit with association constants $K^{ATP}(\beta_1) = 2.0 \times 10^5$ M^{-1} and $K^{ADP}(\beta_1) = 1.4 \times 10^5$ M^{-1}, respectively (Table I). No P_i binding was observed in the absence of Mg^{2+}. The presence of Mg^{2+} results in additional binding of either 1 mol of ATP or ADP with $K^{ATP}(\beta_2) = 5.0 \times 10^3$ M^{-1} and $K^{ADP}(\beta_2) = 1.1 \times 10^4$ M^{-1} (Table I), while Mg^{2+} does not affect the stoichiometry or affinity of nucleotide binding to the Mg-independent site (Gromet-Elhanan & Khananshvili, 1984; Khananshvili & Gromet-Elhanan, 1984). In the presence of Mg^{2+} 0.7–1.0 mol of P_i binds per mole of β to the Mg-dependent site (Khananshvili & Gromet-Elhanan, 1985a,b) with $K^{P}(\beta_2) = 3.7 \times 10^3$ M^{-1}. Under Uni-site conditions the single catalytic site of F_1 binds 1 mol of ATP, ADP, or P_i with $K^{ATP}(F_1) = 1.0 \times 10^{12}$ M^{-1}, $K^{ADP}(F_1) = 3.3 \times 10^6$ M^{-1}, and $K^{P}(F_1) = 1.6 \times 10^3$ M^{-1} (Kasahara & Penefsky, 1978; Cross et al., 1982; Grubmeyer et al., 1982). The fact is that the single catalytic site of F_1 is able to discriminate between ATP and ADP, while neither site of the isolated β-subunit can discriminate between ATP and ADP (Table I).

Scheme I



In order to characterize the involvement of a specific moiety in driving a process, it can be useful to use an empirical definition of "intrinsic binding energy" (Jencks, 1981; Bartlett & Marlowe, 1987; Fersht, 1987). For our concrete situation ΔG^{i} values of the ADP and γ-phosphoryl moieties in the ATP molecule (ΔG^{i}_{ADP} and ΔG^{i}_{P}) as well as ΔG^{i} of the whole molecule of ATP (ΔG^{i}_{ATP}) can be estimated from the observed values of $\Delta G°_{ATP}$, $\Delta G°_{ADP}$, and $\Delta G°_{P}$ according to

$$\Delta G^{i}_{P} = \Delta G°_{ATP} - \Delta G°_{ADP} \quad (2)$$

$$\Delta G^{i}_{ADP} = \Delta G°_{ATP} - \Delta G°_{P} \quad (3)$$

$$\Delta G^{i}_{ATP} = \Delta G^{i}_{ADP} + \Delta G^{i}_{P} \quad (4)$$

Thus, for the Mg-dependent site of β $\Delta G^{i}_{P}(\beta_2) = +0.5$ kcal/mol, $\Delta G^{i}_{ADP}(\beta_2) = -0.2$ kcal/mol, and $\Delta G^{i}_{ATP}(\beta_2) = +0.3$ kcal/mol (Table II). For the Mg-independent site only $\Delta G^{i}_{P}(\beta_1) = +0.3$ kcal/mol can be estimated [it is impossible to estimate $\Delta G^{i}_{ATP}(\beta_1)$ or $\Delta G^{i}_{ADP}(\beta_1)$ because no P_i binding was observed to the Mg-independent site of the isolated β-subunit, see eq 3 and 4]. The fact that the absolute values of all observed intrinsic binding energies are <0.5 kcal/mol

suggests that the isolated β-subunit cannot utilize the intrinsic binding energies of the ADP and γ-phosphoryl moieties effectively for binding of the ATP molecule.

Estimation of ΔG^S *and* ΔG_{12}. The observed binding energy for ATP ($\Delta G°_{ATP}$) can be presented by eq 5, in which (Jencks

$$\Delta G°_{ATP} = \Delta G^i_{ADP} + \Delta G^i_P + \Delta G^S \quad (5)$$

$$\Delta G^S = \Delta G°_{ADP} + \Delta G°_P - \Delta G°_{ATP} \quad (6)$$

1981) a "connection energy", ΔG^S, represents the change in the probability of binding that results from the connection (by a covalent bond) of the separate molecules of ADP and P_i in the whole molecule of ATP. Putting the experimentally measured values of $\Delta G°_{ATP}$, $\Delta G°_{ADP}$, and $\Delta G°_P$ in eq 6, we have $\Delta G^S(\beta_2) = -5.4$ kcal/mol for the Mg-dependent site (Table II). This value of $\Delta G^S(\beta_2)$ is much smaller as compared to that of the single catalytic site of F_1 (Kasahara & Penefsky, 1978; Cross et al., 1982; Grubmeyer et al., 1982), $\Delta G^S(F_1) = +2.9$ kcal/mol.

It was suggested that P_i binds to the γ-phosphoryl binding subsite of ATP in the Mg-dependent site of β (Khananshvili & Gromet-Elhanan, 1985a,b, 1986). The binding of ATP or ADP to the Mg-independent site decreases an apparent dissociation constant for P_i binding from ~250 to ~920 μM (noncompetitive inhibition with $K_I^{ATP} = K_I^{ADP} = 10$ μM), while at high concentrations of ADP the binding of ADP to the Mg-independent site competitively inhibits P_i binding to the γ-phosphoryl binding subsite with $K_I^{ADP} = 10$ mM (Khananshvili & Gromet-Elhanan, 1985a). On the basis of available experimental data, the binding of P_i to the Mg-dependent site of β can be described by Scheme I, in which $K^P = 1.08 \times 10^3$ M^{-1}, $K^{ADP} = 1.1 \times 10^4$ M^{-1}, and $K_I^{ADP} = 100$ M^{-1}. The factor α represents destabilization (reduction) of the binding affinity of ADP to the Mg-dependent site of β in the presence of bound P_i or destabilization of P_i binding in the presence of bound ADP. According to Scheme I, the apparent binding affinity of P_i to the Mg-dependent site of β can be described by eq 7, from which α factor can be estimated by eq 8 (Segel 1975). As can be calculated from the Linew-

$$K^P_{app} = \frac{K^P(1 + \alpha K^{ADP}[ADP])}{1 + K^{ADP}[ADP] + K_I^{ADP}[ADP]} \quad (7)$$

$$\alpha = \frac{K^P_{app}(1 + K^{ADP}[ADP] + K_I^{ADP}[ADP]) - K^P}{K^P K^{ADP}[ADP]} \quad (8)$$

eaver–Burk plot (not shown), in the presence of 1 M ADP the $K^P_{app} = 5.4$ M^{-1}. That gives $\alpha = 4.4 \times 10^{-3}$ (eq 8), which corresponds to $\Delta G_{12}(\beta_2) = +3.4$ kcal/mol ($\Delta G_{12} = -RT \ln \alpha$) (Table II).

Estimation of $\Delta G°_{DP}$, ΔG_I, *and* $\Delta G°_B$. From the definition of $\Delta G°_{DP}$ (Jencks, 1981)

$$\Delta G°_{DP} = \Delta G°_{ADP} + \Delta G°_P + \Delta G_{12} \quad (9)$$

Since $\Delta G°_{ADP}$, $\Delta G°_P$, and ΔG_{12} were measured experimentally for the Mg-dependent site of β (Table II), we can estimate $\Delta G°_{DP}(\beta_2) = -7.1$ kcal/mol (eq 9). This value of $\Delta G°_{DP}(\beta_2)$ is quite close to $\Delta G°_{DP}(F_1) = -8.3$ kcal/mol for the single catalytic site of F_1 (Jencks 1983), suggesting that the difference between two experimental systems in the overall binding energy of ADP + P_i ($\Delta G°_{DP}$) is not so large.

The driving force for the enzyme-bound ATP synthesis can be estimated by the interaction energy, ΔG_I (Jencks, 1981, 1983), which represents the difference for ATP synthesis–hydrolysis in solution ($\Delta G°_N$) and in the enzyme-bound state ($\Delta G°_B$)

$$\Delta G_I = \Delta G°_N - \Delta G°_B \quad (10)$$

or ΔG_I can be presented by equivalent equations

$$\Delta G_I = \Delta G°_{DP} - \Delta G°_{ATP} \quad (11)$$

$$\Delta G_I = \Delta G_{12} + \Delta G^S \quad (12)$$

Thus, from eq 12, $\Delta G_I(\beta_2) = -2.0$ kcal/mol. This value of ΔG_I is very different from the $\Delta G_I(F_1) = +8.0$ kcal/mol (Jencks, 1983). Under experimental conditions similar to those used in this work, ATP synthesis in solution is unfavorable by $\Delta G°_N = +7.6$ kcal/mol (Guynn & Veech, 1973). That allows an estimate of $\Delta G°_B(\beta_2) = +9.6$ kcal/mol from eq 10.

Discussion

The isolated F_1 or the membrane-bound F_0F_1 enzyme can drive enzyme-bound ATP synthesis ($K_{eq} = 0.5$) without external energy input (Boyer, 1979, 1984; Cross et al., 1982; O'Neal & Boyer, 1984; Penefsky, 1985a,b). This is made possible by stronger binding of ATP, $\Delta G°_{ATP}(F_1) = -16.3$ kcal/mol and weak binding of ADP and P_i, $\Delta G°_{DP}(F_1) = -8.3$ kcal/mol. The difference in binding energies of ATP and of ADP + P_i provides a driving force or interaction energy, $\Delta G_I(F_1) = +8.0$ kcal/mol, for the enzyme-bound ATP synthesis, $\Delta G°_B(F_1) = -0.4$ kcal/mol (Jencks, 1983). In the isolated β-subunit, the difference between the $\Delta G°_{ATP}(\beta_2) = -5.1$ kcal/mol and $\Delta G°_{DP}(\beta_2) = -7.1$ kcal/mol cannot support a sufficient interaction energy [$\Delta G_I(\beta_2) = -2.0$ kcal/mol] for the enzyme-bound ATP synthesis, $\Delta G°_B(\beta_2) = +9.6$ kcal/mol (Table II). This indicates that two experimental systems, the isolated β-subunit and the single catalytic site of F_1, show a dramatic difference in the interaction energy ($\Delta\Delta G_I = -10.0$ kcal/mol) and in the enzyme-bound ATP synthesis ($\Delta\Delta G°_B = +10.0$ kcal/mol) (Table II). This situation is caused by the difference in ATP binding ($\Delta\Delta G°_{ATP} = +11.2$ kcal/mol) rather than by the difference in the binding energy of ADP + P_i ($\Delta\Delta G°_{DP} = +1.2$ kcal/mol). A negative value of $\Delta G_I(\beta_2) = -2.0$ kcal/mol (Table II) suggests that the enzyme-bound ATP synthesis–hydrolysis in the isolated β is even more unfavorable than in solution (see eq 10).

The driving force for the enzyme-bound ATP synthesis, ΔG_I, is represented by the sum of the connection energy (ΔG^S) and a linked term (ΔG_{12}) (eq 12). ΔG^S presumably reflects a change in translational and rotational entropy, and ΔG_{12} may represent destabilization by loss of solvation and/or electrostatic repulsion (Jencks, 1975, 1981). For the isolated β-subunit both values, $\Delta G^S(\beta_2) = -5.4$ kcal/mol and $\Delta G_{12}(\beta_2) = +3.4$ kcal/mol, were estimated experimentally (Table II). A measurement of ΔG_{12} in F_1 (under Uni-site catalytic conditions) is difficult because of technical problems (Penefsky, personal communication). However, for F_1, $\Delta G_{12}(F_1) = +5.1$ kcal/mol can be estimated from eq 10 and 12, in which $\Delta G°_B$, ΔG^S, and $\Delta G°_N$ are experimentally measured values in this system. $\Delta G°_{DP}$ is similar in both experimental systems ($\Delta\Delta G°_{DP} = +1.2$ kcal/mol) (Table II); however, Figure 1 shows that a pattern of sequential binding of ADP and P_i is slightly different: Either ADP and Pi can bind and dissociate easily when one ligand is already bound to the Mg-dependent site of the isolated β-subunit [$\Delta G°_P(\beta_2) + \Delta G_{12}(\beta_2) = -1.5$ kcal/mol and $\Delta G°_{ADP} + \Delta G_{12} = -2.2$ kcal/mol] (Figure 1A). In F_1 the binding of P_i to F_1·ADP ($\Delta G°_P + \Delta G_{12} = +0.7$ kcal/mol) is much more unfavorable than the binding of ADP to F_1·P_i ($\Delta G°_{ADP} + \Delta G_{12} = -3.9$ kcal/mol) (Figure 1B). In fact, P_i has a very low affinity to the F_1·ADP complex ($K_P' = 0.2$ M) (Feldman & Sigman, 1982), suggesting that ADP binding to F_1 reduces the binding affinity of P_i ($K_P = 0.14$ mM;

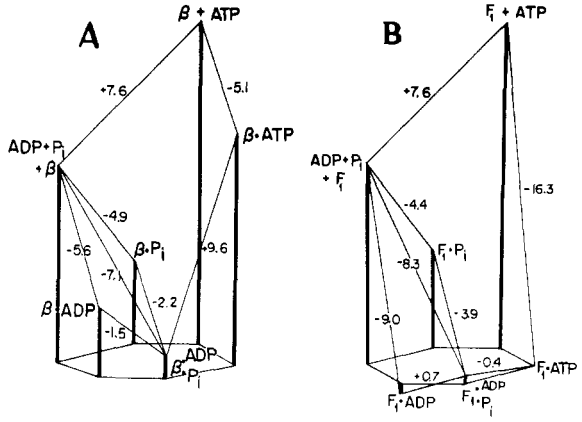


FIGURE 1: Thermodynamic box for ligands binding: (A) enzyme-bound ATP synthesis–hydrolysis and sequential binding of ADP and P_i on the Mg-dependent site of the isolated β-subunit; (B) enzyme-bound ATP synthesis–hydrolysis and sequential binding of ADP and P_i to the single, high-affinity catalytic site of F_1.

Pick & Finnel, 1983) by ~1000-fold. These observations are in good agreement with the estimated value of $\Delta G_{12}(F_1)$ = +5.1 kcal/mol (Table II). A similar value of $\Delta G_{12}(F_1)$ was suggested before (Amzel & Pedersen, 1983). In F_1 both terms, $\Delta G^S(F_1)$ = +2.9 kcal/mol and $\Delta G_{12}(F_1)$ = +5.1 kcal/mol, are important to give a sufficient $\Delta G_I(F_1)$ = +8.0 kcal/mol for favorable enzyme-bound ATP synthesis. In the isolated β-subunit both terms, ΔG^S and ΔG_{12}, have smaller values, $\Delta G^S(\beta_2)$ = −5.4 kcal/mol and $\Delta G_{12}(\beta_2)$ = +3.4 kcal/mol, giving a small value of $\Delta G_I(\beta_2)$ = −2.0 kcal/mol (Table II). Comparison of the ΔG^S and ΔG_{12} values of two experimental systems shows a large value of $\Delta\Delta G^S$ = −8.3 kcal/mol and a relatively small value of $\Delta\Delta G_{12}$ = −1.7 kcal/mol (Table II). This suggests that the contributions of $\Delta\Delta G^S$ and $\Delta\Delta G_{12}$ to the large value of $\Delta\Delta G_I$ = +10.0 kcal/mol or $\Delta\Delta G^\circ_B$ = +10 kcal/mol are not comparable. This fact supports the possibility that the mutual destabilization between bound ADP and P_i (by strain, loss of solvation, and/or electrostatic repulsion) is not changed dramatically upon removing β from F_1, while the isolation of the catalytic β-subunit causes a drastic decrease in the entropy loss term, ΔG^S (by some 8–9 kcal/mol), which causes a drastic decrease in interaction energy for the enzyme-bound ATP synthesis.

F_1 exhibits large absolute values of intrinsic binding energies for both the ADP and the γ-phosphoryl moiety $\Delta G^i_P(F_1)$ = −7.3 kcal/mol, $\Delta G^i_{ADP}(F_1)$ = −11.9 kcal/mol, and $\Delta G^i_{ATP}(F_1)$ = −19.2 kcal/mol, while the Mg-dependent site of the β-subunit fails to do this, $\Delta G^i_{ADP}(\beta_2)$ = −0.2 kcal/mol, and $\Delta G^i_{ATP}(\beta_2)$ = +0.3 kcal/mol (Table II). In F_1 the intrinsic binding energy of ATP, $\Delta G^i_{ATP}(F_1)$ = −19.2 kcal/mol, is manifested to a great extent as observed binding energy, $\Delta G^\circ_{ATP}(F_1)$ = −16.3 kcal/mol, while the overall binding energy of the separate molecules, ADP + P_i [$\Delta G^\circ_{DP}(F_1)$ = −8.3 kcal/mol], is much smaller than the sum of the intrinsic binding energies of the ADP and the γ-phosphoryl moieties [$\Delta G^i_{ADP}(F_1) + \Delta G^i_P(F_1)$ = −19.2 kcal/mol] (Table II). This fact suggests that in F_1 the intrinsic binding energies of ADP and P_i are not expressed in the ground state, but they might be expressed in the transition state. The absolute values of $\Delta\Delta G^S$ = 8.3 kcal/mol, $\Delta\Delta G^i_{ATP}$ = 19.5 kcal/mol, $\Delta\Delta G^i_{ADP}$ = 11.7 kcal/mol, and $\Delta\Delta G^i_P$ = 7.8 kcal/mol (Table II) are all large, suggesting that F_1 (unlike the isolated β-subunit) can utilize the intrinsic binding energy of both moieties, ADP and γ-phosphoryl, for the strong binding of ATP.

The important question is, why is only the F_1 enzyme able to utilize the intrinsic binding energy of the ADP and γ-phosphoryl moieties for the strong binding of ATP in the ground state, while the binding energies of the separate molecules of ADP and P_i are not so different in both, the isolated β and F_1. It was suggested before that each site of β binds a different portion of the nucleotide molecule (Gromet-Elhanan & Khananshvili, 1984; Khananshvili & Gromet-Elhanan, 1985a,b; Scarborough, 1986). It seems likely that the Mg-independent site of β is responsible for the binding of adenosine and/or the sugar moiety of the nucleotide. This stems from the fact that ATP and ADP have an identical pH-dependent pattern for binding in the absence of Mg^{2+}. Furthermore, the binding affinities of these nucleotides are very similar and are not affected by Mg^{2+} at pH 8.0, when all phosphate groups are nearly fully charged (Gromet-Elhanan & Khananshvili, 1984; Khananshvili & Gromet-Elhanan, 1985a). On the other hand, the Mg-dependent site binds ATP, ADP, or P_i only in the presence of Mg^{2+}, suggesting the possibility that phosphoryl-binding subsites are located on this site. Let us assume that two weak binding sites, located on the neighboring β-subunits, form one binding site for ATP (Figure 2). This means that one half-site binds the specific portion of ATP with $\Delta G^\circ_{ATP}(\beta_1)$ = −7.6 kcal/mol (Figure 2A), and the second half-site binds a different specific portion of ATP with $\Delta G^\circ_{ATP}(\beta_2)$ = −5.1 kcal/mol (Figure 2B). In this case the observed binding energy for ATP to F_1 can be presented as $\Delta G^\circ_{ATP}(F_1) = \Delta G^\circ_{ATP}(\beta_{12})$ = −16.3 kcal/mol (Figure 2C). If so, the increase in the binding energy, $\Delta G^S(\beta_{12})$, resulting from the intramolecular binding of ATP can be calculated according to (Jencks, 1981)

$$\Delta G^S(\beta_{12}) = \Delta G^\circ_{ATP}(\beta_1) + \Delta G^\circ_{ATP}(\beta_2) - \Delta G^\circ_{ATP}(\beta_{12}) \quad (13)$$

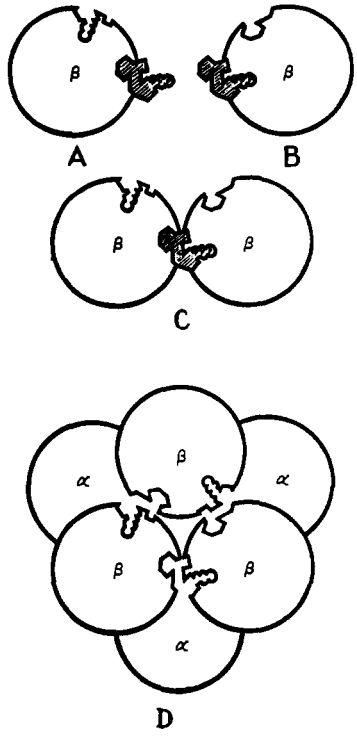


FIGURE 2: Two-half-site model for strong binding of ATP: (A) ATP binding to the Mg-independent site of β with $\Delta G^\circ_{ATP}(\beta_1)$ = −7.6 kcal/mol; (B) ATP binding to the Mg-dependent site of β with $\Delta G^\circ_{ATP}(\beta_2)$ = −5.1 kcal/mol; (C) two-half-site model for strong binding of ATP to the single catalytic site of F_1 with $\Delta G^\circ_{ATP}(\beta_{12})$ = −16.3 kcal/mol [it is proposed that the advantage for the intramolecular binding of ATP is $\Delta G^S(\beta_{12})$ = +3.6 kcal/mol (for explanation see Discussion)]; (D) possible topological resolution of three catalytic sites in the F_1 enzyme (three noncatalytic sites located on the α-subunits are not shown).

The theoretical value of $\Delta G^S(\beta_{12})$ = +3.6 kcal/mol is reasonably close to the value of $\Delta G^S(F_1)$ = +2.9 kcal/mol, which was estimated (see eq 6) by using direct measurements of Penefsky and collaborators (Cross et al., 1982; Grubmeyer et al., 1982). In contrast to ATP the binding of ADP + P_i is weak even in F_1 (Figure 1B), suggesting the possibility that the covalent bond between ADP and P_i provides a maximal loss in translational and rotational entropy (Jencks, 1975, 1981) and overcomes electrostatic repulsion between P_i and ADP. A two-half-site model can explain why two weak binding sites can form one strong binding site for ATP and why the isolation of the β-subunit from F_1 leads to the dramatic changes in $\Delta\Delta G^\circ_{ATP}$, $\Delta\Delta G^\circ_B$, $\Delta\Delta G^S$, $\Delta\Delta G^i_P$, $\Delta\Delta G^i_{ADP}$, $\Delta\Delta G^i_{ATP}$, $\Delta\Delta G_I$, while the changes in $\Delta\Delta G^\circ_{ADP}$, $\Delta\Delta G^\circ_P$, $\Delta\Delta G^\circ_{DP}$, and $\Delta\Delta G_{12}$ are relatively small (Table II).

In the presence of Mg·ATP the isolated β-subunit of *R. rubrum* aggregates to form dimers (M_r 100 000) and/or trimers (M_r 150 000) (Harris et al., 1985). In order to retain a reconstitutive activity of *R. rubrum* β during the long period of isolation and purification procedure, it was necessary to add 2–4 mM Mg·ATP (Mg·ADP cannot prevent inactivation of β) to the buffer solutions (Binder & Gromet-Elhanan, 1974; Philosoph et al., 1977; Khananshvili & Gromet-Elhanan, 1982). It is possible that the aggregated forms of β are more stable in solution than the monomer. This possibility fits with a very well established fact that for the reconstitution of soluble or membrane-bound ATPase activity from the isolated F_1 subunits the presence of millimolar Mg·ATP is necessary (Gromet-Elhanan & Binder, 1974; Philosoph et al., 1977; Kagawa et al., 1978; Futai & Kanazawa, 1983). More detailed investigation is necessary to establish experimental conditions for specific aggregation of β as well as to identify nucleotide binding site, which are involved in this process. This subject might be essential to find a relationship between aggregation phenomena and a possible "weak" ATPase activity (Harris et al., 1985).

A two-half-site model is consistent also with the accumulated experimental data of photoaffinity labeling; various photoaffinity analogues of nucleotides can covalently bind to two different domains of catalytic β-subunit in the assembled F_1 (Kironde & Cross, 1987; Cross et al., 1987; Xue et al., 1987). Strongest argument for the close orientation of catalytic β-subunits in F_1 was provided by Joshi and Wang (1987). They have shown that a specific cross-linking of adjacent β-subunits in F_1 through the residues of Lys 162 and Glu 199 (that are thought to be at the catalytic site) gives a dimer of β-subunits, suggesting that only two copies of β-subunits are close to each other in F_1 (the length of cross-linking reagent is 4–5 Å). A proper approximation of the two catalytic half-sites to each other may control an operation of negative cooperativity between the catalytic sites during the multiple turnover of the enzyme. According to the two-half-site model, the only difference between the high- and low-affinity catalytic sites of F_1 is that the high-affinity catalytic site can provide a spontaneous intramolecular binding of ATP, while the low-affinity catalytic (promotor) site needs some approximation of two half-sites for strong binding of ATP. The remaining F_1 subunits may control an approximation between two catalytic half-sites, which might play a crucial role in the regulation of negative cooperativity (Melese & Boyer, 1985; Wang, 1985).

It was shown that $\Delta\mu H^+$ can release strongly bound ATP from the single catalytic site of the membrane-bound F_0F_1 complex (Penefsky, 1985a). That means that $\Delta\mu H^+$ = 8–11 kcal/mol can release strongly bound ATP with ΔG^i_{ATP} = −19.2 kcal/mol. Therefore, $\Delta\mu H^+$ cannot provide enough energy for complete destabilization of binding subsites that are involved in the binding of specific moieties of strongly bound ATP. On the other hand, any destabilization of these subsites could decrease ΔG_I, resulting in reversion of ATP to ADP + P_i on the catalytic site of the enzyme. In order to avoid this, a requirement of the system is to release ATP rapidly (Boyer, 1979, 1984; Jencks, 1980, 1983) before it is converted back to ADP and P_i. This kinetic requirement may provide a specific mechanism for destabilization of binding subsites in β. For example, in order to "save" ATP from hydrolysis, it is reasonable to postulate that $\Delta\mu H^+$ destabilizes the phosphoryl-binding subsite(s), causing a fast displacement of the phosphoryl moieties of ATP. As a consequence, the ATP molecule may still bind to the adenosine/ribose (Mg-independent site) binding half-site, from which it may dissociate in solution in an "energy-independent" manner. Dissociation of ATP from this half-site is perhaps a rate-limiting step in the overall release of bound ATP.

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