concerning the specific contribution of $\Delta\psi$ to the driving force for uphill Na^+ efflux at acidic pHs. Their data would also be of interest to analyze whether kinetic and thermodynamic aspects of the antiport function can be related to each other.

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Characterization of Two Nucleotide Binding Sites on the Isolated, Reconstitutively Active β Subunit of the F_0·F_1 ATP Synthase†

Zippora Gromet-Elhanan* and Daniel Khananshvili

ABSTRACT: The reconstitutively active β subunit that has been removed from the *Rhodospirillum rubrum* membrane-bound ATP synthase (RrF_0·F_1) by LiCl extraction [Philosoph, S., Binder, A., & Gromet-Elhanan, Z. (1977) *J. Biol. Chem. 252*, 8747–8752] and purified to homogeneity [Khananshvili, D., & Gromet-Elhanan, Z. (1982) *J. Biol. Chem. 257*, 11377–11383] binds both ATP and ADP. In the absence of $MgCl_2$ 1 mol of ATP or ADP is bound per mol of β subunit with K_d values of 4.4 μM and 6.7 μM, respectively. The binding of both nucleotides is optimal at a pH range between 7.6 and 8.3 and has a characteristic half-time of 3 min. Addition of $MgCl_2$ has no influence on the stoichiometry, kinetics, or affinity of the binding of ADP to β. It has also no effect on the binding of ATP at limiting concentrations. But at high ATP concentrations, an additional binding site, which is dependent on the concentration of both ATP and $MgCl_2$, is revealed. Thus, a maximal binding stoichiometry of 2 mol of ATP per mol of β subunits is obtained with ≥2 mM ATP at a $MgCl_2$ to ATP ratio of at least 0.5. Under these conditions, one ATP molecule binds to the β with a K_d and $t_{1/2}$ identical with those recorded in the absence of $MgCl_2$, whereas the second ATP molecule binds with a K_d of 200 μM and a $t_{1/2}$ of 20 min. These results indicate that the isolated β subunit of the RrF_0·F_1 ATP synthase contains two nucleotide binding sites: one that binds either ATP or ADP and is independent on the presence of $MgCl_2$ and one that binds rather specifically ATP in the presence of $MgCl_2$.

The terminal step of ATP synthesis in energy-transducing membranes is generally accepted to be catalyzed by a membrane-bound reversible proton-translocating ATPase (Mitchell, 1966), which has been isolated from membranes of mitochondria, bacteria, and chloroplasts and found to consist of two portions: F_1 and F_0 (Penefsky, 1979; Fillingame, 1981; Nelson, 1981). The catalytic F_1 portion is an extrinsic membrane protein composed of five different subunits: α, β, γ, δ, and ϵ. The F_0 portion is an intrinsic membrane complex that

† From the Department of Biochemistry, The Weizmann Institute of Science, Rehovot 76100, Israel. *Received June 29, 1983.*

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contains at least three subunits and mediates proton-translocation across the membrane. The molecular mechanism of ATP synthesis and hydrolysis is still unknown, although a number of mechanisms have been proposed (Boyer et al., 1977; Cross, 1981). One possible approach to the elucidation of this problem is to study the ligand binding sites located on the F_0·F_1 (Harris, 1978; Baird & Hammes, 1979; Jencks, 1980; Shavit, 1980). Previous studies have shown that the catalytic F_1 portion has several nucleotide binding sites that reside in the two larger subunits, α and β. Thus, modification of F_1 by alkylating (Drusta et al., 1979; Esch & Allison, 1979) or photoaffinity (Cosson & Guillory, 1979; Carlier et al., 1979; Wagenvoord et al., 1979; Lunardi et al., 1981) analogues of adenine nucleotides results in labeling of both α and β. Up to six nucleotide binding sites have been observed on the whole F_1 (Cross & Nalin, 1982), and they seem to include both catalytic and regulatory sites. However, the subunit location of these sites is still uncertain. It is, therefore, most interesting to examine possible nucleotide binding sites on isolated, reconstitutively active α and β subunits and compare their properties with those recorded for the proposed catalytic and regulatory sites on the whole F_1.

Reconstitutively active α and β subunits have been obtained up to now only from bacterial sources. From a thermophilic bacterium (Yoshida et al., 1977) and from *Escherichia coli* (Futai, 1977), all five subunits have been separated by dissociating their F_1 complexes, and ATPase activity could be reconstituted by reassociation of mixtures of purified α, β, and γ. In *Rhodospirillum rubrum*, two subunits, β and γ, have been removed from the membrane-bound RrF_0·F_1[1] (Philosoph et al., 1977, 1981; Gromet-Elhanan et al., 1981) and purified to homogeneity (Khananshvili & Gromet-Elhanan, 1982). Both ATP synthesis and hydrolysis activities of the depleted membranes have been fully restored after rebinding the purified subunits. These individually isolated and purified subunits provide an ideal system for investigating possible nucleotide binding sites since, although none of them has any catalytic activity by itself (Futai, 1977; Philosoph et al., 1977; Yoshida et al., 1977), they retain the capacity to restore ATP synthesis and/or hydrolysis after their reconstitution.

Direct binding studies with either labeled ADP and ATP or various analogues have been carried out mainly on the α subunit, since in most cases their binding to the β subunit could not be detected [for a review, cf. Futai & Kanazawa (1983)]. Indirectly, nucleotide binding to the β subunit has been suggested from the effect of added ATP and ADP on the circular dichroism spectrum of β (Ohta et al., 1980) and from the effect of ATP on the increased fluorescence of anilinonaphthalenesulfonate in the presence of β (Futai & Kanazawa, 1983). There is only one report on the photolabeling of the isolated β subunit of EcF_1 with a photoaffinity ADP analogue (Lunardi et al., 1981), which resulted in the binding of $\sim$1 mol of photolabel/mol of β subunit after three successive photoirradiation cycles. However, no data on ATP binding are presented, and no further characterization of the ADP binding site has been attempted.

In this investigation, we report studies on the direct binding of ADP and ATP to the purified, reconstitutively active β subunit of RrF_1. Our results indicate that this β subunit has two nucleotide binding sites showing widely different kinetic and affinity properties.

Experimental Procedures

R. rubrum cells were grown as outlined by Philosoph et al. (1977) and chromatophores prepared by the Yeda press according to Gromet-Elhanan (1970, 1974), except that they were suspended in 50 mM Tricine–NaOH (pH 8.0), 0.25 M sucrose, 5 mM $MgCl_2$, and 10 μg/mL deoxyribonuclease and ribonuclease (Khananshvili & Gromet-Elhanan, 1982), washed twice in 50 mM Tricine–NaOH (pH 8.0) and 0.5 mM EDTA, and finally suspended in 1 mM Tricine–NaOH plus glycerol (1:1 v/v). The reconstitutively active β subunit of RrF_0·F_1 was isolated from *R. rubrum* chromatophores, purified, and stored as previously described (Khananshvili & Gromet-Elhanan, 1982). In all experiments reported here, an electrophoretically pure β subunit, which restored 90–95% of the photophosphorylation or Mg^{2+}-ATPase activities of β-less chromatophores, was used. Before incubation of the β subunit with any adenine nucleotide, it was freed from the storage buffer, which contains ATP, $MgCl_2$, and glycerol, by elution–centrifugation (Penefsky, 1977) in a Sephadex G-50 column equilibrated with 100 mM Tricine–NaOH, pH 8.0, as outlined by Khananshvili & Gromet-Elhanan (1983). The elution–centrifugation for buffer exchange, as well as for the removal of unbound nucleotides (see below), was carried out at 4 °C.

Binding studies were carried out by incubating ^{3}H-labeled adenine nucleotides with the β subunit at 10 μM in 10 mM Tricine–NaOH, pH 8.0, at 23 °C under the conditions described in the figure legends. Incubation was initiated by addition of the β subunit, and at the specified time intervals the unbound nucleotides were removed by subjecting 50-μL samples to elution–centrifugation on Sephadex columns preequilibrated with 10 mM Tricine–NaOH, pH 8.0. In order to enable a simultaneous collection of a number of samples without changing the time of exposure of the sample to residual buffer in the column, the method introduced by Cross & Nalin (1982) was modified as follows: The samples were placed in an Eppendorf yellow (200-μL) tip, which was inserted on the top of each Sephadex column prepared in a 1-mL tuberculin syringe. In this way, the sample did not enter the column until centrifugation was initiated. After all samples were placed in the tips, the columns were cooled for 10 min at 4 °C and centrifuged. The effluent from each column was assayed for ^{3}H radioactivity and protein content. When samples containing 0.5–3.0 mg of β/mL were loaded on the column, the recovery of protein in the column effluent was above 90% (see Figure 2). In control experiments, carried out without β, less than 0.005% of the applied ^{3}H radioactivity appeared in the effluent.

Protein concentration was determined according to Lowry et al. (1951). ^{3}H radioactivity was measured by liquid scintillation counting according to Penefsky (1977). Binding data were calculated by using a molecular weight of 50 000 for the β subunit (Bengis-Garber & Gromet-Elhanan, 1979). The dissociation constants were obtained by treatment of Scatchard plots according to Rosenthal (1967).

[2,8-^{3}H]ATP (23–29 Ci/mmol) and [2,8-^{3}H]ADP (28–30 Ci/mmol) were obtained from New England Nuclear. Nonradioactive ATP and ADP were obtained from Sigma, purified by ion-exchange chromatography (Cohn & Carter, 1950), and concentrated by lyophilization. The radioactive ATP and ADP were added to the respective nonradioactive nucleotides to give a specific radioactivity of 130–150 Ci/mol,

[1] Abbreviations: RrF_0·F_1, proton-translocating ATP synthase–ATPase complex of *R. rubrum*; RrF_1, soluble *R. rubrum* ATPase; EcF_1, soluble *E. coli* ATPase; MF_1, soluble mitochondrial ATPase; TF_1, soluble thermophilic bacterium PS3 ATPase; Tricine, *N*-[tris(hydroxymethyl)-methyl]glycine; Hepes, 4-(2-hydroxyethyl)-1-piperazineethansulfonic acid; Mes, 2-(*N*-morpholino)ethanesulfonic acid; EDTA, ethylenediaminetetraacetic acid.

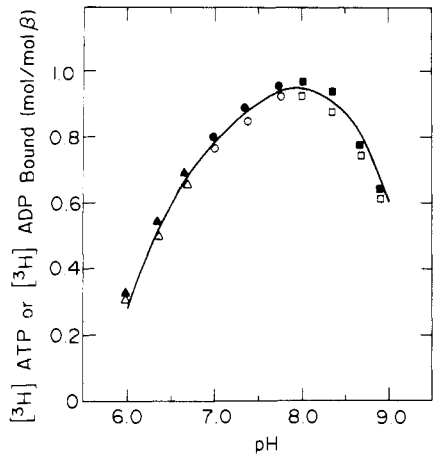

FIGURE 1: Effect of pH on binding of ATP and ADP by the β subunit. The β subunit at 0.5 mg/mL was equilibrated by elution–centrifugation with 100 mM of the following buffers: Mes–NaOH (▲, △), Hepes–NaOH (●, ○), or Tricine–NaOH (■, □) at the indicated pH. 0.12 mL of effluent was incubated in 10 mM of the above buffers with 4 mM [^{3}H]ATP (▲, ●, ■) or 4 mM [^{3}H]ADP (△, ○, □) for 1 h. Aliquots of 50 μL were freed from unbound nucleotides by elution–centrifugation on Sephadex G-50 columns equilibrated with 10 mM of the specified buffers and assayed for ^{3}H radioactivity and protein content as described under Experimental Procedures.

which was used in all experiments.

Results

Binding of ATP and ADP to the β Subunit in the Absence of $MgCl_2$. The β subunit is isolated, purified, and stored in the presence of ATP and $MgCl_2$ (Philosoph et al., 1977; Khananshvili & Gromet-Elhanan, 1982) since it has been found to lose its reconstitutive capacity on their removal (Binder & Gromet-Elhanan, 1974). But examination of the nucleotide binding sites on a reconstitutively active β is dependent on establishing conditions for removal of previously bound ATP and $MgCl_2$ without losing the reconstitutive capacity. While studying the chemical modification of the β subunit, Khananshvili & Gromet-Elhanan (1983) have shown that its reconstitutive activity is retained for several hours when it is incubated in Tricine–NaOH, pH 8.0 with no other additions at 4 or 20 °C and on addition of 20% glycerol even at 30 °C. In order to circumvent possible effects of glycerol on the binding properties of β, all binding experiments were carried out by incubating the β subunit in buffer without glycerol at 23 °C.

The ability of β to bind nucleotides was examined by the elution–centrifugation technique. Both ADP and ATP were bound, showing a similar pH dependence (Figure 1). The binding was optimal between pH 7.6 and 8.3 when about 0.90–0.95 mol of either ATP or ADP was bound per mol of β, so all further experiments were run at pH 8.0. A very similar range of optimal pH was shown for various photophosphorylation systems (Briller & Gromet-Elhanan, 1970; Feldman & Gromet-Elhanan, 1971), as well as for ATP hydrolysis (Horio et al., 1965) in *R. rubrum* chromatophores. On the other hand, an optimal pH of 7.0 was observed by Dunn & Futai (1980) for binding of ATP by the purified reconstitutively active α subunit of EcF_1.

The effect of varying concentrations of β on the binding stoichiometry of ATP and ADP was tested (Figure 2) with saturating concentrations of the adenine nucleotides (see Figures 5 and 6). Their binding stoichiometry was constant at β concentrations ranging between 2 and 50 μM. At concentrations below 10 μM, the recovery of β from the Sephadex column decreased drastically, although the binding stoichiometry of the recovered protein remained unchanged (Figure 2). β at 10 μM (=0.5 mg/mL) was therefore used in all further experiments, since this is the minimal protein concentration that is fully recovered after elution–centrifugation. A similar minimal protein concentration of 0.5 mg/mL has also been shown to be required for full recovery of the whole beef heart F_1 in the column effluents (Cross & Nalin, 1982). It has been reported that the purified, reconstitutively active β subunit of EcF_1 tends to form dimers and higher aggregates (Dunn & Futai, 1980). We have no information on the possible formation of aggregates of the purified RrF_1 β, but even if such aggregates are formed, the results of Figure 2 indicate that they could not change the observed binding stoichiometry of the adenine nucleotides in the absence of $MgCl_2$.

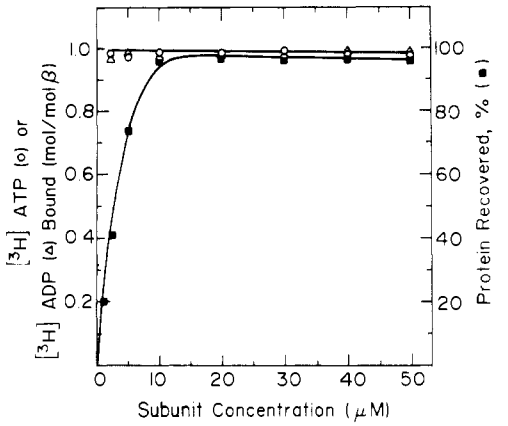

FIGURE 2: Effect of concentration of β subunit on its recovery from the Sephadex columns and on binding stoichiometry of the adenine nucleotides. The indicated concentrations of β subunit were incubated in 10 mM Tricine–NaOH, pH 8.0, with 4 mM ATP (○) or 4 mM ADP (△) for 1 h. Aliquots of 50 μL were freed from unbound nucleotides and assayed for bound nucleotides as described in Figure 1. Protein recovery is given as percentage of the protein content in the samples loaded on the column. The binding stoichiometry was calculated from the ^{3}H radioactivity and protein content determined in the effluent.

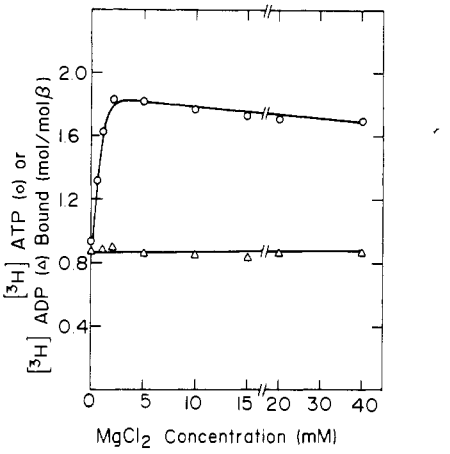

FIGURE 3: Effect of $MgCl_2$ on the ATP and ADP binding sites of the β subunit. β was incubated at 10 μM in 0.12 mL of 10 mM Tricine–NaOH, pH 8.0, containing 4 mM ATP (○) or 4 mM ADP (△) and the indicated concentrations of $MgCl_2$ under the conditions specified in Figure 1.

Binding of ATP and ADP to the β Subunit in the Presence of $MgCl_2$. The effect of $MgCl_2$ on the nucleotide binding sites was tested by adding increasing concentrations of $MgCl_2$ to an incubation mixture containing saturating concentrations of ATP and ADP (Figure 3). Addition of $MgCl_2$ did not influence at all the binding of ADP, which, in the presence of varying $MgCl_2$ concentrations, reached the same maximal

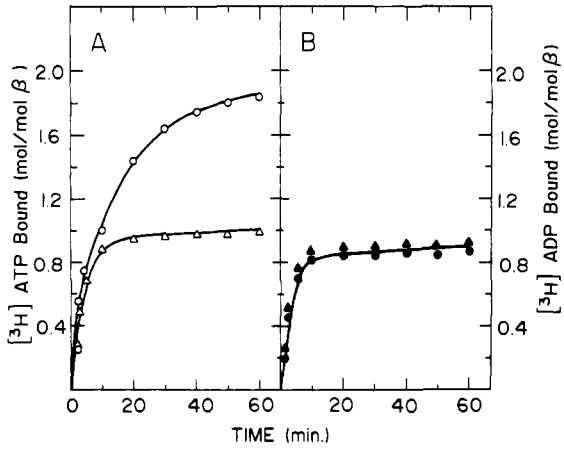

FIGURE 4: Time dependence of ATP (A) and ADP (B) binding to the β subunit. β was incubated at 10 μM in 10 mM Tricine–NaOH, pH 8.0, with either 4 mM ATP (○, △) or 4 mM ADP (●, ▲) without (△, ▲) or with (○, ●) 2 mM $MgCl_2$ in a total volume of 2 mL. At the time indicated, 50-μL aliquots from each incubation were freed from unbound nucleotides and assayed for bound nucleotides as described in Figure 1.

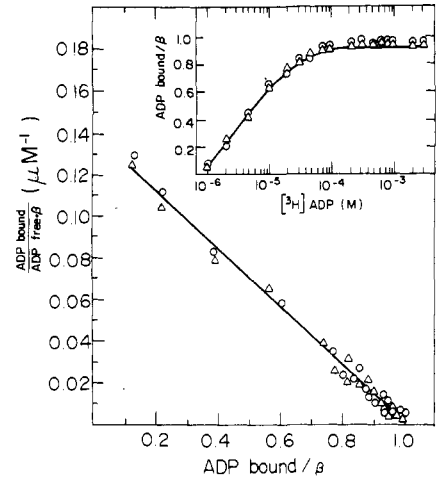

FIGURE 5: Scatchard plot analysis of ADP binding to the β subunit. β was incubated at 10 μM in 0.12 mL of 10 mM Tricine–NaOH, pH 8.0, for 2 h with the indicated concentrations of [^{3}H]ADP in the absence (○) or presence (△) of $MgCl_2$. $MgCl_2$ was added at concentrations that gave a ratio of Mg^{2+} to ADP of 0.5 over the whole concentration range of ADP. Aliquots of 50 μL were freed from unbound ADP and assayed for bound ADP as described in Figure 1.

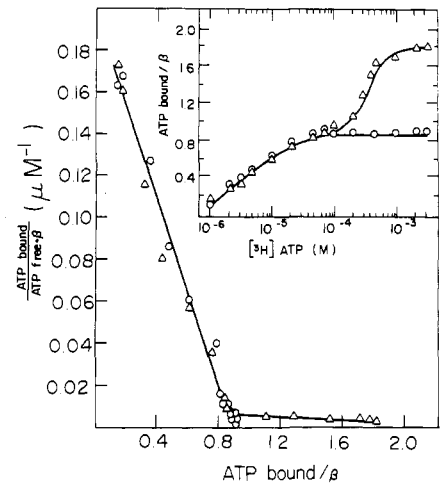

FIGURE 6: Scatchard plot analysis of ATP binding to the β subunit. Incubation and assay conditions were as described in Figure 5.

level of ~0.9 mol of ADP bound/mol of β recorded in the absence of $MgCl_2$ (see also Figures 1 and 2). With ATP, on the other hand, an additional binding site was revealed in the presence of $MgCl_2$ (Figure 3). Thus, its binding to the β subunit increased linearly with increasing $MgCl_2$ concentrations from the basic level of ~0.9 mol/mol of β observed in the absence of $MgCl_2$ to a maximal level of ~1.8 mol of ATP bound/mol of β obtained at 2 mM $MgCl_2$ when the Mg^{2+} to ATP ratio was 0.5. This maximal level of binding stoichiometry was not affected by a further increase in the $MgCl_2$ concentration, leading to Mg^{2+} to ATP ratios of up to 10.

The time dependence of nucleotide binding to the β subunit is illustrated in Figure 4. In the absence of $MgCl_2$, the binding of both ATP and ADP reached the maximal level of 0.9–1.0 mol/mol of β at about 10 min with a $t_{1/2}$ of 3 min (Figure 4). Addition of $MgCl_2$ had no effect on the kinetics of ADP binding, which showed the same $t_{1/2}$ in the absence and presence of $MgCl_2$. The presence of $MgCl_2$ had, however, a marked influence on the kinetics of ATP binding, which became biphasic (Figure 4A). The first rapid phase is very similar to that observed in the absence of $MgCl_2$, resulting in the binding of ~0.9 mol of ATP/mol of β, whereas the second phase, which leads to binding to 1 additional mol of ATP/mol of β, is much slower, reaching equilibrium binding only after 1 h with a $t_{1/2}$ of about 20 min. These data suggest the existence of a second binding site on the β subunit that is specific for ATP, is dependent on the presence of Mg^{2+} ions, and has different kinetic properties from the Mg^{2+}-independent site that binds both ATP and ADP.

The existence of two nonidentical binding sites for ATP on β was clearly demonstrated in experiments where β was incubated, without and with $MgCl_2$, in the presence of a wide range of nucleotide concentrations for 2 h, in order to enable even the slow binding of ATP in the presence of $MgCl_2$ to reach equilibrium (see insets to Figures 5 and 6). With ADP, about 1 mol of binding site/mol of β was titrated in both the absence and presence of $MgCl_2$, showing a monophasic binding curve that saturates at 0.1 mM ADP. With ATP, a very similar monophasic binding curve was observed in the absence of $MgCl_2$, but in the presence of $MgCl_2$, a biphasic binding curve was observed. The first phase was identical with that observed in the absence of $MgCl_2$ (and with that obtained with ADP under all conditions tested), whereas the second phase saturated only around 2 mM ATP, reaching a maximal binding of about 1.8 mol of ATP/mol of β. A Scatchard plot analysis of the binding data (Figures 5 and 6) reveals only one binding site for ADP with a K_d of 6.6 μM, both with and without $MgCl_2$ (Figure 5), but two binding sites for ATP (Figure 6), one high-affinity site with a K_d of 4.4 μM, similar to the ADP binding site that is independent on $MgCl_2$, and one low-affinity site with a K_d of 200 μM that operates only in the presence of $MgCl_2$. This K_d is similar to the K_m for ATP hydrolysis of 130 μM measured by Horio et al. (1971) in *R. rubrum* chromatophores and of 200–400 μM measured by Futai et al. (1974) in the EcF_1 ATPase, so this site might be a catalytic one.

The presence of at least 2 mM Mg-ATP, which cannot be replaced by Mg-ADP (Binder & Gromet-Elhanan, 1974), has been previously shown to be essential for preservation of full reconstitutive activity in the RrF_1 β subunit during its isolation, purification, and storage (Philosoph et al., 1977; Khananshvili & Gromet-Elhanan, 1982). Our findings that this purified β subunit has a low-affinity site that binds specifically ATP in the presence of $MgCl_2$ suggest that in order to keep the β subunit in an active form, this site must be occupied. For reconstitution of this active β into β-less chromatophores, a much higher $MgCl_2$ concentration of 20–25 mM is required

(Gromet-Elhanan et al., 1981). The binding of ATP to the low-affinity site on β does not require such a high Mg^{2+} to ATP ratio of 5 but is also not affected by it (see Figure 3). We can, therefore, conclude that this binding site remains occupied throughout the reconstitution period and that the high $MgCl_2$ concentration is required for the rebinding of the β into the β-less chromatophores rather than for the binding of Mg-ATP to the isolated β subunit.

Discussion

Two types of nucleotide binding sites, differing in their affinity and kinetic properties, have been identified on F_1 ATPases from various sources (Harris, 1978; Baird & Hammes, 1979; Slater et al., 1979; Shavit, 1980). These nucleotide binding sites have been found to reside in the α and β subunits, and it has therefore been suggested that each of these subunits contains one type of binding sites. Direct tests of this suggestion became possible when isolated purified α and β subunits, which retain their native active form, became available (Futai, 1977; Philosoph et al., 1977; Yoshida et al., 1977).

The experiments reported in this paper demonstrate that the homogeneously purified β subunit of RrF_1, which retains full reconstitutive activity (Khananshvili & Gromet-Elhanan, 1982), contains two nucleotide binding sites having different kinetic and affinity properties. One is a high-affinity site that is independent on $MgCl_2$ and binds either ATP or ADP with a K_d of 4–7 μM and a $t_{1/2}$ of 3 min. The second is a low-affinity site that is dependent on $MgCl_2$ and binds only ATP with a K_d of 200 μM and a $t_{1/2}$ of 20 min.

The only other report where a nucleotide–β subunit complex has been isolated and its binding stoichiometry measured is that of Lunardi et al. (1981), which used the same elution–centrifugation technique employed here. They have followed the binding of a photoaffinity [^{3}H]ADP analogue to the β subunit of EcF_1 after a number of successive cycles of photoirradiation, but only in the absence of $MgCl_2$. Under these conditions, they found a binding stoichiometry of $\sim$1 mol of photolabel/mol of β that was markedly decreased by prior incubation with ADP or ATP, indicating that the labeled site can bind either ADP or ATP. This nucleotide binding site on the EcF_1 β subunit is thus similar to the high-affinity binding site reported here in the RrF_1 β subunit that binds ADP or ATP in a $MgCl_2$-independent manner. Lunardi et al. (1981) present no information on the effect of $MgCl_2$ on nucleotide binding to the EcF_1 β subunit. Therefore, further experiments will be required to examine whether the EcF_1 β subunit contains also the second, Mg-ATP specific, low-affinity binding site that we have demonstrated in the RrF_1 β subunit. An indication for the presence of such a low-affinity binding site for ATP has recently been reported by Futai et al. (1982). They detected binding by following changes in the fluorescence of anilinonaphthalenesulfonate, which increased upon addition of the isolated EcF_1 β subunit. This increased fluorescence was quenched upon addition of 0.1–1 mM ATP (Futai & Kanazawa, 1983), which is identical with the concentration range required for demonstration of the second low-affinity binding site for ATP on the RrF_1 β subunit (Figure 6).

Two additional methods, equilibrium dialysis and gel filtration, have been used for isolation of nucleotide–subunit complexes with subunits isolated from EcF_1 or TF_1 (Dunn & Futai, 1980; Matsuoka et al., 1982; Ohta et al., 1980). They did provide information on the nucleotide binding properties of the α subunit but not of the β subunit. Thus, when measured by equilibrium dialysis, the α subunit of EcF_1 was found to contain one high-affinity binding site that could bind [^{3}H]ATP, [^{3}H]ADP (Dunn & Futai, 1980), or a fluorescent [^{32}P]ATP analogue (Matsuoka et al., 1982), but no binding of these nucleotides by the EcF_1 β subunit was detected. The ligand concentrations used by these authors with either the α or β subunits ranged from 0.1 to 2 μM. In light of our observations (see Figures 5 and 6) that even the high-affinity binding site on RrF_1 β requires 0.1 mM ADP or ATP for saturation and the low-affinity Mg-ATP site does not saturate below 1 mM ATP, it is unlikely that these sites would be detected by the equilibrium dialysis measurements.

Ohta et al. (1980) have tried to measure direct nucleotide binding to α and β isolated from TF_1 by trapping the nucleotide–subunit complexes on nitrocellulose membrane filters after binding of [^{3}H]ADP or [^{3}H]ATP. They found binding of both nucleotides to both subunits but failed to isolate stoichiometric (1:1) nucleotide–subunit complexes. They, therefore, assayed the nucleotide binding indirectly by following changes in the circular dichroism spectra of TF_1 and its purified α and β subunits on addition of the nucleotides. But, this assay does not provide data on the binding stoichiometry of the nucleotides to the subunits, so for calculating K_d values a certain stoichiometry had to be assumed. With an assumed 1:1 stoichiometry for nucleotide binding to the TF_1 β, Ohta et al. (1980) obtained K_d values of 8.5 μM and 15 μM for ADP and ATP, respectively, which were not significantly changed by $MgCl_2$. These K_d values are quite similar to those obtained here for the Mg-independent high-affinity binding site. Their calculations do not provide information on the possible existence of a second, low-affinity, nucleotide binding site on TF_1 β. Interestingly, the K_d values of 120 μM for ADP and 18 μM for ATP calculated by Ohta et al. (1980) for the TF_1 α subunit are quite different from those measured with the EcF_1 α by Dunn & Futai (1980), which were 0.9 μM for ADP and 0.1 μM for ATP. It is not clear at present whether the differences stem from the completely different methods employed in these studies or reflect the different sources of the isolated subunits.

Assuming that the nucleotide binding sites of the α and β subunits isolated from different F_1 ATPase are similar, the results obtained up to now indicate that the isolated α subunit contains one nucleotide binding site, whereas the isolated β subunit has two binding sites (Figure 7, part I). The single binding site on the isolated α subunit is a high-affinity site that binds either ATP or ADP with K_d values that are typical to tight regulatory binding sites (Dunn & Futai, 1980; Ohta et al., 1980; Lunardi et al., 1981). One of the two binding sites observed on the isolated β subunit is also a high-affinity site that binds either ATP or ADP and is not affected by $MgCl_2$ [Figures 6; see also Ohta et al. (1980) and Lunardi et al. (1981)], but the second site on β has completely different properties. It is a low-affinity site that binds only ATP in the presence of $MgCl_2$ [Figures 3, 4, and 6; see also Futai et al. (1982)] with a K_d value of 200 μM (Figure 6), which is similar to the K_m for ATP hydrolysis (Horio et al., 1971; Futai et al., 1974). So this site might be the catalytic one.

These binding sites exist probably also when the α and β subunits are assembled in the F_1 ATPase, since it is not likely that the solubilization and purification procedures lead to the formation of new nucleotide binding sites. We do not know, however, whether all these sites operate independently when the subunits are assembled in the catalytic F_1 complex, and therefore, they should not be employed as evidence for any specific $\alpha\beta$ stoichiometry. One possible situation where, in the assembled state, only two nucleotide binding sites are operating per each pair of α and β subunits is presented in Figure 7, part II. On the basis of the similar properties of the

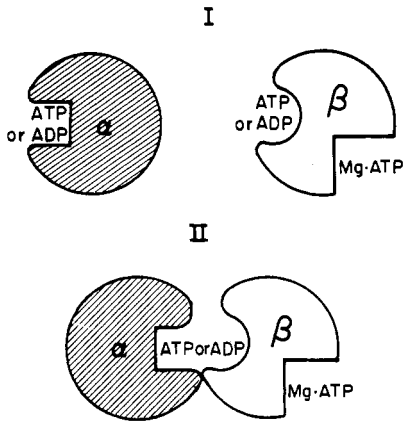

FIGURE 7: Stoichiometry of nucleotide binding sites on the isolated α and β subunits and on an assembled $\alpha\beta$ complex of the F_1 ATPase. Part I shows the three nucleotide binding sites that have been observed on isolated α and β subunits. Part II proposes a possible arrangement of these sites when the α and β subunits are assembled in the F_1 ATPase in a way that leads to a functional operation of two distinct nucleotide binding sites per each pair of α and β subunits. One is the Mg^{2+}-dependent low-affinity binding site for ATP that is located on the β subunit. The second is a Mg^{2+}-independent high-affinity binding site for ATP and ADP that is located at the interface between the assembled α and β subunits.

high-affinity binding sites found in the isolated α and β subunits, it shows one high-affinity site located between a pair of assembled α and β subunits. Each subunit might interact with a different portion of the nucleotide molecule such as, for instance, the adenine ring and the α- and β-phosphoryl oxygens. Indeed, our results suggest that the adenosine moiety is more important for interaction with the high-affinity binding site on the β subunit than the charge on the phosphoryl group. Since, although at the pH used here (8.0), which is optimal for their binding (Figure 1), the adenine nucleotides are nearly fully charged, addition of Mg^{2+}, which decreases their charge, has no influence on the rate of binding or the affinity of ADP and ATP at this high-affinity binding site (Figures 4–6). After dissociation of the F_1, each isolated subunit might retain its binding properties, thus leading to the appearance of the three distinct nucleotide binding sites illustrated in Figure 7, part I.

This suggestion could explain some seemingly contradictory reports dealing with the labeling of various F_1 ATPases by 8-azido-ATP. After denaturation and gel electrophoresis, the label appeared mainly in the β subunit of MF_1 (Wagenvoord et al., 1977) but in the α subunit of EcF_1 (Verheijen et al., 1978). Both groups measured the labeling in the absence of $MgCl_2$, because in its presence the nucleotide was hydrolyzed by the F_1 ATPases. This ATP analogue could, therefore, bind in both F_1 ATPases at the high-affinity sites shared by the α and β subunits, and after dissociation it could be retained by either α or β or both, depending on the conditions used for denaturation and gel electrophoresis. In light of these possibilities, it will be most interesting to compare the nucleotide binding sites of the isolated α and β subunits with those of an $\alpha\beta$ complex assembled from the same subunits. Formation of an $\alpha\beta$ subunit complex from the isolated subunits has been reported up to now only with the TF_1 α and β subunits (Kagawa & Nukiwa, 1981).

The interpretation presented in Figure 7, part II, which suggests that an adenine nucleotide binding site is situated at an interface between the α and β subunits, has been proposed by Cosson & Guillory (1979) and later by others [see Senior & Wise (1983) for a comprehensive review]. However here, unlike in previous models, this binding site is not suggested to have a catalytic role. It is rather proposed to be the high-affinity binding site for ADP and ATP that is independent on Mg^{2+} and is not catalytic but could be regulatory.

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Registry No. ATP, 56-65-5; ADP, 58-64-0; Mg, 7439-95-4; ATP synthase, 37205-63-3.

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