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Articles

Regulation of $\Delta\tilde{\mu}_H$+-Coupled ATP Synthesis and Hydrolysis: Role of Divalent Cations and of the F_0F_1-β Subunit†

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ABSTRACT: The divalent cation specificity of ATP-linked reactions catalyzed by the H^+-translocating F_0F_1 ATP synthase–ATPase complex has been followed in the *Rhodospirillum rubrum* chromatophore bound complex. In the presence of Mg^{2+} and Mn^{2+} the complex catalyzes ATP synthesis and hydrolysis as well as ATP-driven H^+ translocation, but in the presence of Ca^{2+} it catalyzes only ATP hydrolysis, which is not coupled to H^+ tanslocation. The inability of Ca^{2+} to maintain the coupling process is not due to opening of a proton leak in its presence nor to any release of F_1 from the membrane, because (a) an identical light-induced H^+ translocation is observed in the absence or presence of Ca·ATP and (b) the Ca-ATPase, as well as the Mg- and Mn-ATPase activities, is blocked by specific F_0 inhibitors. These results indicate that the divalent cations play an important role in the regulation of H^+-coupled ATP synthesis and hydrolysis by the F_0F_1 complex. Further tests suggest that their site of action is located on the F_1-β subunit. The isolated β subunit of the *R. rubrum* F_0F_1 has been reported to contain two nucleotide binding sites, a Mg-independent and a Mg-dependent site [Gromet-Elhanan, Z., & Khananshvili, D. (1984) *Biochemistry 23*, 1022–1028]. Addition of Mn^{2+} also enables the binding of 2 mol of ATP/mol of this isolated β subunit. But under identical conditions, Ca^{2+} does not enable ATP binding to this cation-dependent site and inhibits its binding in the presence of Mg^{2+} or Mn^{2+}. In light of these results we propose that the binding of Mg·ATP or Mn·ATP, but not of Ca·ATP, to the *R. rubrum* F_1-β subunit forms the trigger that opens the pathway for H^+ translocation through the F_0F_1 complex during catalysis.

Electron-transport coupled ATP synthesis in respiratory and photosynthetic organisms is catalyzed by a reversible proton-translocating ATP synthase–ATPase complex that is composed of two distinct structures (Senior & Wise, 1983; Merchant & Selman, 1985): a readily solubilized catalytic ATPase sector, F_1, and an intrinsic membrane sector that is involved in proton fluxes, F_0. The F_1-ATPase from many different sources has a $\alpha_3\beta_3\gamma\delta\epsilon$ subunit structure and contains

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the catalytic site(s) for ATP synthesis, although in its soluble state it catalyzes only ATP hydrolysis. It is also involved in proton translocation during catalysis, namely, proton-coupled ATP synthesis and ATP-driven proton uptake, which are catalyzed only by the membrane-bound whole F_0F_1 complex.

The detailed mechanism of coupling between photosynthetic or respiratory electron transport, proton translocation, and ATP synthesis is as yet unsolved (Mitchell, 1981, 1985; Ferguson, 1985). Some old as well as recent data indicate that divalent cations play a role in this coupling process. Thus, coupled photophosphorylation in plant thylakoid membranes has been shown to require the presence of Mg^{2+} ions that could be replaced by Co^{2+}, but not by Mn^{2+} or Ca^{2+} (Whatly et al., 1959). ATP hydrolysis on the other hand occurs in the presence of either Mg^{2+} or Ca^{2+}, but it has recently been reported that the chloroplast-bound Ca-ATPase, unlike its Mg-ATPase, is not coupled to proton translocation (Pick & Weiss, 1988). These results point to a strict correlation between the capacity of a divalent cation to catalyze photophosphorylation and to induce ATP-driven proton translocation.

In chromatophore membranes of the photosynthetic bacterium *Rhodospirillum rubrum*, unlike in chloroplasts, some photophosphorylation has been observed at low Ca^{2+} concentrations (Edwards & Jackson, 1976). We have therefore assayed in this work the divalent cation specificity of ATP synthesis, hydrolysis, and ATP-driven proton uptake in these chromatophores. Our results have revealed that Mg^{2+} and Mn^{2+} catalyze all three reactions, whereas Ca^{2+} catalyzes only ATP hydrolysis, which is not coupled to proton translocation.

The observed specific behavior of the native *R. rubrum* chromatophore bound F_0F_1 (RrF_0F_1)[1]–enzyme complex in the presence of Ca^{2+} ions is very similar to that of the hybrid F_0F_1 complexes, formed by reconstitution of β subunits isolated from *Escherichia coli* (Ecβ) or chloroplast F_1-ATPase ($CF_1\beta$) into β-less *R. rubrum* chromatophores (Gromet-Elhanan et al., 1985b; Richter et al., 1986). Unlike the native enzyme complex, these hybrids exhibit, even in the presence of Mg^{2+} ions, a rather low photophosphorylation activity and a high ATPase activity, which is not coupled to proton uptake. Furthermore, the chloroplast/chromatophore hybrid has a high specificity for Mg·ATP as substrate for ATP hydrolysis, showing no Ca-ATPase activity (Richter et al., 1986). The results with the hybrids have suggested that the F_1-β subunit is involved in ATP-coupled proton translocation as well as in conferring the different metal ion specificity upon the F_0F_1–ATP synthase complex.

In this work we have, therefore, also attempted to define the site responsible for the divalent cation specificity on the F_1-β subunit, using the isolated, purified *R. rubrum* β subunit, Rrβ (Philosoph et al., 1977; Khananshvili & Gromet-Elhanan, 1982). Occupation of the cation·ATP binding site that has earlier been characterized on Rrβ (Gromet-Elhanan & Khananshvili, 1984) has been found to be induced by Mn^{2+} as well as Mg^{2+}, but inhibited by Ca^{2+}. Our results with *R. rubrum* chromatophores as well as Rrβ suggest that the cation·ATP binding site on the F_1-β subunit plays an important role in regulating the capacity of the F_0F_1 enzyme complex to catalyze $\Delta\tilde{\mu}_{H^+}$-coupled ATP synthesis and hydrolysis.

[1] Abbreviations: CF_0F_1 and RrF_0F_1, proton-translocating ATP synthase–ATPase complex of chloroplasts and *Rhodospirillum rubrum*; $CF_1\beta$, Ecβ, and Rrβ, isolated, reconstitutively active β subunit of the F_0F_1 complex of chloroplasts, *Escherichia coli*, and *R. rubrum*; Bchl, bacteriochlorophyll; EDTA, ethylenediaminetetraacetic acid; Tricine, *N*-[tris(hydroxymethyl)methyl]glycine; DCCD, dicyclohexylcarbodiimide.

Materials and Methods

Materials. [2,8-^{3}H]ATP (30–35 Ci/mmol) was obtained from New England Nuclear and diluted with chromatographically purified nonradioactive ATP (Hackney et al., 1980) to give a specific radioactivity of 15–40 Ci/mol. [^{32}P]P_i was obtained from the Nuclear Research Center, Negev.

Preparation of R. rubrum Chromatophores and Rrβ. The chromatophores used throughout this investigation were prepared as described by Gromet-Elhanan and Khananshvili (1986), washed three times in buffer containing 0.5 mM EDTA to remove endogenous $MgCl_2$, and stored in 10 mM Tricine–NaOH, pH 8.0, plus glycerol (1:1 v/v) in liquid nitrogen. Reconstitutively active Rrβ was isolated, purified, and stored as outlined by Gromet-Elhanan and Khananshvili (1986). Before investigating the binding of ATP to Rrβ, it was freed from the ATP and $MgCl_2$ present in its storage buffer as described by Gromet-Elhanan and Khananshvili (1984), except that the Sephadex G-50 columns were preequilibrated with TGN buffer containing 50 mM Tricine–NaOH, pH 8.0, 20% glycerol, and 50 mM NaCl.

Analytical Procedures. Photophosphorylation was carried out as previously described (Gromet-Elhanan et al., 1985b), except that glucose and hexokinase were omitted and varying concentrations of divalent cations were used as indicated. Formation of [γ-^{32}-P]ATP was measured according to Avron (1960).

ATPase activities were assayed in a reaction mixture containing, in a final volume of 0.7 mL, 50 mM Tricine–NaOH, pH 8.0, chromatophores containing 4 μg of bacteriochlorophyll, and varying concentrations of divalent cations as indicated in the text. After 10 min of equilibration at 35 °C ATP hydrolysis was started by addition of 4 mM ATP and stopped after 10 min by 0.1 mL of 2 M trichloroacetic acid. The released P_i was measured according to Taussky and Shorr (1953).

Light-dependent and ATP-dependent quenching of quinacrine (atebrin) fluorescence was determined as described by Gromet-Elhanan et al. (1985b), except that $MgCl_2$ was omitted from the initial illuminated reaction mixture.

ATP binding to Rrβ was carried out by incubating [^{3}H]ATP with the β subunit at 10 μM in buffer containing 50 mM Tricine–NaOH, pH 8.0, and 20% glycerol without or with the indicated varying concentrations of divalent cations. The incubation was initiated by addition of β subunit, and after 2 h at 23 °C the mixture was subjected to elution–centrifugation on Sephadex G-50 columns, preequilibrated with TGN buffer, to separate the free and bound [^{3}H]ATP, as described by Gromet-Elhanan and Khananshvili (1984).

Protein was determined either by the procedure of Lowry et al. (1951) or by the method of Bradford (1976) using bovine serum albumin as the standard. Bacteriochlorophyll was measured by using the in vivo extinction coefficient given by Clayton (1963). ^{3}H radioactivity was assayed by liquid scintillation counting according to Penefsky (1977). Binding data were calculated by using a molecular weight of 50 000 for the Rrβ (Bengis-Garber & Gromet-Elhanan, 1979; Falk et al., 1985).

Results

Divalent Cation Specificity of ATP Synthesis and Hydrolysis. Photophosphorylation by the EDTA-washed *R. rubrum* chromatophores is fully dependent on addition of divalent cations (Figure 1). ATP synthesis is catalyzed in the presence of either Mg^{2+} or Mn^{2+}, but Mg^{2+} is about 2-fold more effective. The rate of photophosphorylation increases

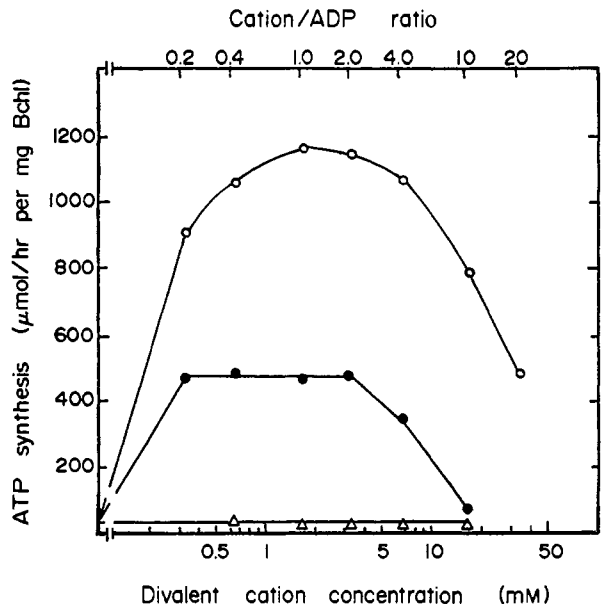

FIGURE 1: Divalent cation requirement of photophosphorylation by *R. rubrum* chromatophores. The reaction mixture contained, in a final volume of 1 mL, 50 mM Tricine–NaOH, pH 8.0, 2 mM ADP, 4 mM sodium phosphate containing about 5×10^6 cpm of ^{32}P, 66 μM *N*-methylphenazonium methosulfate, chromatophores containing 4–5 μg of bacteriochlorophyll, and the indicated concentrations of $MgCl_2$ (○), $MnCl_2$ (●), or $CaCl_2$ (△).

linearly with the cation concentration, reaching saturation at about 0.3–1.0 mM (giving a cation/ADP ratio of 0.2–0.6). There is a rather wide optimal cation concentration, but at 20 mM Mn^{2+} completely inhibits ATP synthesis, whereas Mg^{2+} inhibits it by about 50%. Whatley et al. (1959) have tested the effet of increasing Mg^{2+} and Mn^{2+} concentrations on photophosphorylation in chloroplasts. Their observed Mg stimulation was very similar to its effect in chromatophores, reaching saturation at the same Mg/ADP ratios. Mn^{2+} on the other hand was ineffective in chloroplasts, when tested at the same range of concentrations.

As is illustrated in Figure 1, Ca^{2+} does not enable ATP synthesis at any concentration. Edwards and Jackson (1976) have observed a low rate of photophosphorylation at 1 mM Ca^{2+}, which was inhibited by 5 mM Ca^{2+}, in *R. rubrum* chromatophores. Their chromatophores were, however, prepared in buffer containing 8 mM $MgCl_2$, which was omitted only from the final resuspension medium. So their "Ca-dependent photophosphorylation" was probably due to the presence of enough residual $MgCl_2$.

ATP hydrolysis, unlike its synthesis, is carried out by *R. rubrum* chromatophores in the presence of all three cations, with Mn^{2+} being even somewhat more effective than Mg^{2+} (Figure 2). The activity increased linearly with the cation concentration, and with Mg^{2+} or Mn^{2+} it reached a maximal level at a cation/ATP ratio of 0.5, decreasing at high ratios. With Ca^{2+}, on the other hand, a lower rate of ATPase activity was obtained, but it continued to increase up to a cation/ATP ratio of 2.0, so that at this ratio a very similar ATPase activity was observed with all three tested cations (Figure 2).

It has earlier been shown that the cation·ATP complex is the substrate for ATP hydrolysis in chromatophores and that excess free Mg^{2+} behaves as a competitive inhibitor (Oren & Gromet-Elhanan, 1979). The above results indicate that excess free Mn^{2+} is an even more efficient inhibitor of ATP hydrolysis than Mg^{2+}, whereas Ca^{2+} is not, although it does inhibit photophosphorylation (Whatly et al., 1959; Edwards & Jackson, 1976).

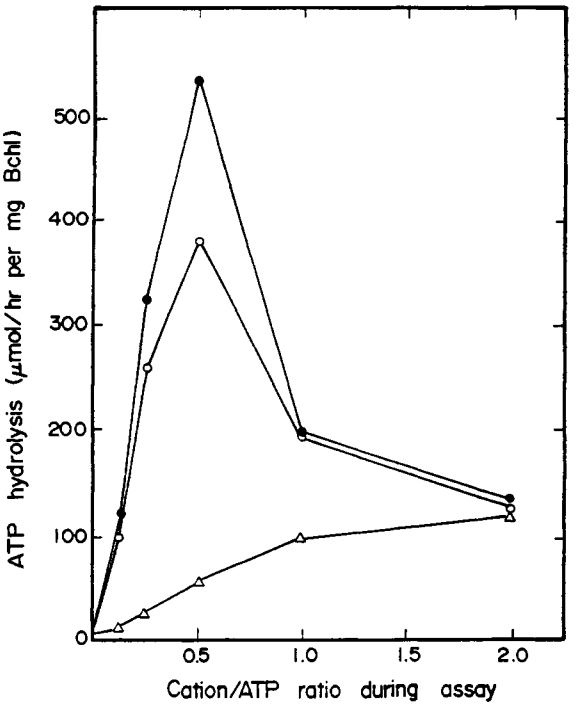

FIGURE 2: Divalent cation requirement of ATP hydrolysis by *R. rubrum* chromatophores. Reaction conditions were as described under Materials and Methods using 4 mM ATP and varying concentrations of $MgCl_2$ (○), $MnCl_2$ (●), or $CaCl_2$ (△) to give the indicated cation/ATP ratios.

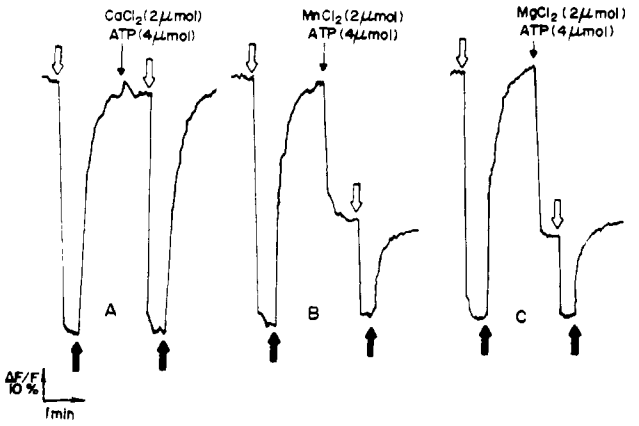

FIGURE 3: Effect of divalent cations on light- and ATP-dependent quenching of quinacrine fluorescence by *R. rubrum* chromatophores. Assay conditions were as described under Materials and Methods. The reaction mixture contained, in a final volume of 2.7 mL, 50 mM Tricine–NaOH, pH 8.0, 50 mM KCl, 1 μM valinomycin, 0.2 mM diaminodurene, and chromatophores containing 8–10 μg of bacteriochlorophyll. White and black arrows indicate light on and off, respectively. Where indicated, 80 μL of a mixture containing 50 mM ATP and 25 mM of the indicated divalent cations were added.

Effect of Divalent Cations on Light- and ATP-Driven Proton Translocation. The Mg- and Mn-ATPase activity of *R. rubrum* chromatophores is coupled to proton translocation. This is illustrated in Figure 3 by following the quenching of quinacrine fluorescence. The light-induced quenching has been shown to reflect the light-induced proton uptake by these chromatophores (Gromet-Elhanan, 1977). Addition of either Mg·ATP (Figure 3B) or Mn·ATP (Figure 3C) results also in a quenching of quinacrine fluorescence, which amounts to about 60% of the light-induced quench and is increased to its maximal level by further illumination in the presence of these cation·ATP complexes. Unlike the Mg- or Mn-ATPase activities of *R. rubrum* chromatophores, their Ca-ATPase activity is not coupled to proton translocation (Figure 3A).

Table I: Dependence of ATP-Induced Quenching of Quinacrine Fluorescence on the Cation/ATP Ratio[a]

	ATP-induced quenching of fluorescence in the presence of		
cation/ATP ratio	$MgCl_2$	$CaCl_2$	$MnCl_2$
0.12	25	5[b]	21
0.25	55	6	50
0.50	60	6	60
1.00	45	5[b]	54
1.50	22	5[b]	20

[a] Light-induced quenching and ATP-induced quenching were carried out as described in Figure 3, except that the ATP-induced quenching was started by addition of 80 μL of 25 mM ATP containing varying concentrations of the indicated divalent cations yielding the desired cation/ATP ratios. The numbers represent the ATP-induced quench as percent of the light-induced one. They are an average of five experiments using different chromatophore preparations, whose ATPase activities varied by less than 20%. [b] Five percent of the light-induced quench is the limit of detection, which includes fluctuations caused by the injection of 80 μL of a solution containing only ATP.

The inability of Ca^{2+} to maintain a proton-coupled ATPase activity is not due to any general uncoupling effect of Ca^{2+} because, as is shown in Figure 3A, the same degree of light-induced quenching of quinacrine fluorescence was obtained when the chromatophores were illuminated in the absence or presence of Ca·ATP. Furthermore, the effect of Ca^{2+} is not due to any Ca-dependent release of the F_1-ATPase from the chromatophore membrane, because the Ca-ATPase activity of *R. rubrum* chromatophores has been shown to be as sensitive as their Mg-ATPase activity to both oligomycin (Oren & Gromet-Elhanan, 1977) and DCCD (Oren & Gromet-Elhanan, 1979). These two energy-transfer inhibitors have also been found to inhibit the chromatophores' Mn-ATPase activity (not shown).

The above results have been recorded with a cation/ATP ratio of 0.5 (Figure 3), which is optimal for the Mg- and Mn-ATPase activities, but not for the Ca-ATPase (Figure 2). The cation specificity of the ATPase-driven quenching of quinacrine fluorescence was, therefore, compared at various cation/ATP ratios (Table I) that cover a wide range of ATPase activities. The results show that the Ca-ATPase did not induce a significant quench at any tested Ca/ATP ratio, whereas the Mg- and Mn-ATPase induced a marked quench at all tested ratios. This difference holds also for cation/ATP ratios that sustain very similar rates of ATP hydrolysis, such as 0.12 for Mg^{2+} and Mn^{2+} and 1.0–2.0 for Ca^{2+} (see Figure 2).

These results indicate that the inability of Ca^{2+} to maintain ATP synthesis and ATP-driven proton translocation must be an inherent property of the native membrane-bound F_0F_1 Ca-ATPase. Hybrid F_0F_1 complexes, obtained by reconstituting β-less *R. rubrum* chromatophores with Ecβ or $CF_1\beta$, have been found to behave in the presence of Mg^{2+} very similarly to the native RrF_0F_1 in the presence of Ca^{2+} (Gromet-Elhanan et al., 1985b; Richter et al., 1986). Moreover, as with the native Ca-ATPase, the very low capacity of the hybrid Mg-ATPase to maintain an ATP-driven H^+ translocation has also been found as *not* due to any hybrid-induced proton leak or F_1 release from the membrane (Gromet-Elhanan et al., 1985b). It thus seems that the divalent cations as well as the F_1-β subunit play an important role in the regulation of H^+-coupled ATP synthesis and hydrolysis. Furthermore, their effects seem to be interrlated, since the F_0F_1 hybrid, containing $CF_1\beta$, has no Ca-ATPase activity (Richter et al., 1986), while in the native RrF_0F_1 all three cations have been found capable of inducing ATP hydrolysis (Figure 2).

Binding of ATP to Rrβ in the Presence of Divalent Cations.

Table II: Effect of Various Divalent Cations on ATP Binding to the Isolated, Reconstitutively Active *R. rubrum* F_1-β Subunit[a]

	ATP bound (mol/mol of β) in the presence of		
cation/ATP ratio	$MgCl_2$	$MnCl_2$	$CaCl_2$
0.5	1.8	1.8	0.5
1.0	2.0	1.0	0.4
2.0	0.8	0.5	0.2

[a] Binding was carried out as described under Materials and Methods with 1 mM [^{3}H]ATP and divalent cations at the indicated cation/ATP ratios, in a final volume of 0.12 mL. After 2 h at 23 °C aliquots of 50 μL were freed from unbound ATP by elution–centrifugation on Sephadex G-50 columns. The effluent from each column was diluted with 0.5 mL of water, and appropriate aliquots were assayed for ^{3}H radioactivity and protein content.

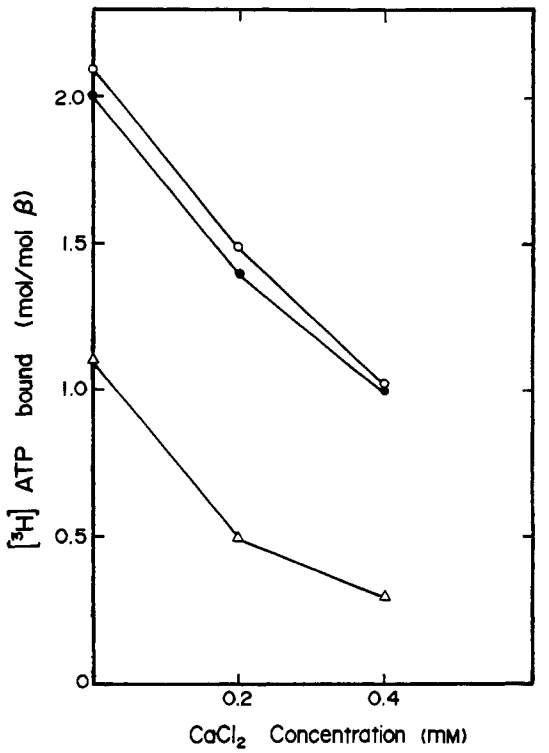

FIGURE 4: Inhibition of ATP binding to Rrβ by $CaCl_2$. ATP binding was carried out as described in Table II, but with 0.4 mM [^{3}H]ATP, without (△) or with 0.2 mM $MgCl_2$ (○) or $MnCl_2$ (●), and the indicated concentrations of $CaCl_2$.

We have earlier reported that the isolated, reconstitutively active Rrβ contains two nucleotide binding sites: a high-affinity Mg-independent site and a low-affinity Mg-dependent site (Gromet-Elhanan & Khananshvili, 1984; Khananshvili & Gromet-Elhanan, 1984). In light of the above results the divalent cation specificity of ATP binding to Rrβ has been determined (Table II). Here too Mn^{2+} is very similar to Mg^{2+}, enabling the binding of 2 mol of ATP/mol of Rrβ, when added at a cation/ATP ratio of 0.5. Also, in this binding assay (Table II), as in ATP synthesis and hydrolysis (Figures 1 and 2), excess free Mn^{2+} is more inhibitory than free Mg^{2+}. Thus, at a cation/ATP ratio of 1.0, ATP binding in the presence of Mn^{2+} has decreased to 1 mol/mol of Rrβ, whereas in the presence of Mg^{2+} it remained at 2 mol/mol of Rrβ.

Ca^{2+} again behaves differently than Mg^{2+} and Mn^{2+}. It does not enable, at any cation/ATP ratio, the binding of ATP to its second cation-dependent site, as demonstrated by elution–centrifugation on Sephadex G-50 columns (Table II). Moreover, as is illustrated in Figure 4, Ca^{2+} inhibits ATP binding to its first as well as second binding sites. When the binding was assayed with Mg^{2+} or Mn^{2+} at the optimal cat-

ion/ATP ratio of 0.5, addition of Ca^{2+} at a 2-fold higher concentration decreased ATP binding from 2 to 1 mol/mol of Rrβ.

The consistently different behavior of Ca^{2+} as compared to Mg^{2+} and Mn^{2+} in all our tests, including ATP binding to Rrβ, suggests that the divalent cations might affect the regulation of H^+-coupled ATP synthesis and hydrolysis by the F_0F_1–enzyme complex via the cation-dependent ATP binding site of the F_1-β subunit.

Discussion

Mg^{2+} and Ca^{2+} have been reported to exert different effects on the thylakoid membrane bound CF_0F_1 ATP synthase–ATPase complex. Thylakoids carry out Mg-dependent ATP synthesis (Whatley et al., 1959) and hydrolysis (Petrack et al., 1965), and the Mg-ATPase is coupled to H^+ translocation (Carmeli, 1970). Ca^{2+} on the other hand maintains only ATP hydrolysis (Whatley et al., 1959; Avron, 1962), which has recently been characterized as not coupled to H^+ translocation (Pick & Weiss, 1988). In chromatophores of the photosynthetic bacterium *Rhodobacter capsulatus* similar results, namely, that only their Mg-ATPase activity is coupled to generation of $\Delta\tilde{\mu}_{H^+}$, have recently been reported (Casadio, 1988).

The latent CF_0F_1-ATPase activity is, however, dependent on proper activation, and different optimal conditions have been established for activation of the Ca- as compared to the Mg-ATPase activities [see Pick and Weiss (1988)]. The *R. capsulatus* chromatophores had to be prepared in buffers containing either $CaCl_2$ or $MgCl_2$, to preserve the binding of F_1 to F_0, and the Ca chromatophores were reported to contain around 2 mM Mg^{2+} (Casadio, 1988). For detailed assays of the divalent cation specificity of coupled ATP synthesis and hydrolysis it was therefore important to find a membrane-bound F_0F_1 that (a) can be thouroughly washed in EDTA to remove all traces of free Mg^{2+} and (b) is capable of hydrolyzing ATP without any prior activation. The *R. rubrum* chromatophores used here have been shown to fulfill both requirements. Their washing, or even sonication, in EDTA buffer did not release the F_1 from their membrane-bound F_0F_1 complex (Gromet-Elhanan, 1974). Also, their Ca- and Mg-ATPase activities have been shown to be independent on prior activation in the light, as long as a low cation/ATP ratio was used (Oren & Gromet-Elhanan, 1979; Gromet-Elhanan et al., 1985a).

The experiments reported in this paper (Figures 1–3) indicate that Mg^{2+} and Ca^{2+} affect the *R. rubrum* chromatophore bound RrF_0F_1 in a similar manner to their effect on the chloroplast-bound CF_0F_1. Taken together, these results suggest the existence of a close correlation between the divalent cation requirement for photophosphorylation and H^+-coupled ATP hydrolysis. Indeed, the observed effect of Mn^{2+} on RrF_0F_1 (Figures 1–3 and Table I) provides further evidence for this correlation.

The effect of Mn^{2+} on the chloroplast-bound CF_0F_1 is as yet unclear. Whatley et al. (1959) were unable to demonstrate any Mn-dependent photophosphorylation. The effect of Mn^{2+} on coupled ATP hydrolysis has not been tested, but from experiments with the soluble CF_1 it seems to behave much more similarly to Mg^{2+} than to Ca^{2+} (Hochman et al., 1976; Hiller & Carmeli, 1988). It will therefore be most interesting to carry out a detailed characterization of the effect of Mn^{2+} as compared to Ca^{2+} and Mg^{2+} on H^+-coupled ATP synthesis and hydrolysis by the chloroplast-bound CF_0F_1.

The results observed with the native RrF_0F_1 in the presence of Ca^{2+} are similar to those observed with hybrid F_0F_1 complexes even in the presence of Mg^{2+} (Gromet-Elhanan et al., 1985b; Richter et al., 1986). The main difference between the native RrF_0F_1 and these hybrids is in their β subunit, since they were constructed by reconstitution of β-less *R. rubrum* chromatophores with Ecβ or $CF_1\beta$. Thus the inability of the Ca-ATPase of native RrF_0F_1 and the Mg-ATPase of the hybrid F_0F_1 complexes to drive H^+ translocation suggests that the β subunit as well as the divalent cations are involved in the regulation of H^+-coupled ATP synthesis and hydrolysis.

An indication that the effects of the divalent cations and the $F_1\beta$ subunits can be connected has been obtained from experiments with the isolated, reconstitutively active Rrβ. The Mg-dependent ATP binding site that has earlier been demonstrated on this F_1-β subunit (Gromet-Elhanan & Khananshvili, 1984) was found to bind ATP also in the presence of Mn^{2+}, but not of Ca^{2+} (Table II). Moreover, Ca^{2+} rather inhibited the binding of ATP in the presence of Mg^{2+} or Mn^{2+} (Figure 4).

It should be emphasized that the chromatophore-bound RrF_0F_1, as well as the isolated whole RrF_0F_1 complex, and the RrF_1 are all active Ca-ATPase (Oren & Gromet-Elhanan, 1979; Bengis-Garber & Gromet-Elhanan, 1979; Johansson et al., 1973). Furthermore, the cation·ATP complex has been shown to be the substrate for ATP hydrolysis in chloroplasts (Hochman et al., 1976) and in *R. rubrum* chromatophores (Oren & Gromet-Elhanan, 1979). Ca·ATP should therefore bind to the F_1-β subunit. The inability of Ca^{2+} to induce ATP binding to the isolated Rrβ, when assayed by elution–centrifugation through Sephadex G-50 columns, does not mean that Rrβ cannot bind Ca·ATP, but that the binding parameters of Ca·ATP are different from those of Mg·ATP or Mn·ATP. If for instance Ca·ATP dissociates much faster than either Ma·ATP or Mn·ATP from Rrβ, its binding would not be detected by this method.

Different binding parameters could induce different conformational states on the F_1-β subunit. Ligand-induced changes in conformation of the F_1-β subunit could be the trigger for opening or closing the pathway of H^+ translocation through the F_0F_1 complex during catalysis. Ligand binding has been shown to induce two different types of changes in the conformation of Rrβ, which were reflected in its sensitivity to partial proteolysis (Khananshvili & Gromet-Elhanan, 1986). One conformational state was obtained on occupation of the cation-independent nucleotide binding site and was further stabilized by Mg·ADP. A second, very different conformational state was obtained on binding of Mg·ATP or Mg·P_i. A comparison of the effects of ATP and P_i in the presence of Mg^{2+}, Mn^{2+}, and Ca^{2+} on the conformational state of Rrβ is now under investigation.

If Mn·ATP and Mn·P_i will behave also in the proteolysis assay as the Mg complexes, whereas the Ca complexes will be different, it would provide direct evidence for our suggestion that the cation-dependent nucleotide binding site on Rrβ (and on the F_1-β subunits in general) plays an important role not only in the catalytic activity of the F_0F_1–enzyme complex but also in the regulation of the coupling process. According to this suggestion, occupation of this site on Rrβ by Mg·ATP or Mn·ATP, but not by Ca·ATP, induces a conformational change that results in opening of the pathway for H^+ translocation through RrF_0F_1 during catalysis.

Registry No. ATPase, 9000-83-3; Mg·ATP, 1476-84-2; Mn·ATP, 56842-80-9; ATP synthase, 37205-63-3.

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Identification of the Membrane Anchor of Microsomal Rat Liver Cytochrome P-450†

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ABSTRACT: Cytochrome P450IIB1 isolated from rat liver microsomes was incorporated into phosphatidylcholine/phosphatidylethanolamine/phosphatidylserine (10:5:1 w/w) liposomes. Trypsinolysis of proteoliposomes and sequencing of the membrane-bound domains revealed that only one peptide, comprising amino acid residues 1–21, spans the membrane. Modification of the N-terminal methionine by membrane-impermeable fluorescein isothiocyanate occurred with the protein in solution but not in proteoliposomes. We conclude that in proteoliposomes cytochrome P-450 spans the membrane only with amino acid residues 1–21, the N-terminal methionine facing the lumen.

The microsomal monooxygenase system comprises several membrane-bound proteins. NADPH–cytochrome P-450 reductase is inserted in the membrane via a 6-kilodalton (kDa)[1] N-terminal peptide, which spans the bilayer twice. A water-soluble protein can be set free with protease, the protein being then unable to interact with cytochrome P-450 (P-450) (Gum & Strobel, 1981). Cytochrome b_5 spans the membrane only once with its C-terminus, the localization of the last amino acid being unclear (Tennyson & Holloway, 1986; Arinc et al., 1987). The membrane topology of P-450, the terminal and key enzyme of the microsomal monooxygenase system, is not known with certainty. Models in which a large part of the protein is imbedded in the bilayer were proposed on the basis

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[1] Abbreviations: DPH, diphenylhexatriene; FITC, fluorescein isothiocyanate; kDa, kilodalton(s); P-450, cytochrome P-450; PC, phosphatidylcholine; PE, phosphatidylethanolamine; PS, phosphatidylserine; r^s, steady-state fluorescence anisotropy; SDS, sodium dodecyl sulfate.

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