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Division of Divalent Cations into Two Groups in Relation to Their Effect on the Coupling of the F_0F_1-ATPase of *Rhodospirillum rubrum* to the Protonmotive Force†

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Received December 8, 1988; Revised Manuscript Received July 28, 1989

ABSTRACT: Divalent cations are divided into two groups in relation to their ability to promote ATP synthase catalyzed reactions. In the presence of Mg^{2+}, the following pattern rules: (i) uncoupler-stimulated ATP hydrolysis of *Rhodospirillum rubrum* chromatophores which shows an optimum concentration of the divalent cation; (ii) ATP-induced proton pumping in chromatophores; (iii) light-induced ATP synthesis in chromatophores; (iv) no or very low ATPase activity of purified F_1-ATPase unmasked by diethylstilbestrol or *n*-octyl β-D-glucopyranoside. In the presence of Ca^{2+}, the following pattern occurs: (i) no stimulation of the ATP hydrolysis in chromatophores by carbonyl cyanide *p*-(trifluoromethoxy)phenylhydrazone; (ii) no ATP-induced proton pumping; (iii) no light-induced ATP synthesis; (iv) a high ATPase activity of the purified F_1-ATPase which is inhibited by diethylstilbestrol and *n*-octyl β-D-glucopyranoside. Co^{2+}, Mn^{2+}, and Zn^{2+} are members of the "Mg^{2+}-group", whereas Cd^{2+} is suggested to fall between the two groups. Intrinsic uncoupling of the membrane-bound ATP synthase has been suggested to account for the effect caused by Ca^{2+} in chloroplasts [Pick, U., & Weiss, M. (1988) *Eur. J. Biochem. 173*, 623-628]. Such an interpretation is consistent with our results on chromatophores. The uncoupling cannot occur at the level of the membrane since neither light-induced nor Mg-ATP-induced proton pumping is affected by Ca^{2+}. A conformational change is suggested to be the reason for this intrinsic uncoupling, and it is proposed to be controlled by the diameters of the divalent cations ($Ca^{2+} > Cd^{2+} > Mn^{2+} > Co^{2+} > Zn^{2+} > Mg^{2+}$). Furthermore, the members of the "Mg^{2+}-group" and Cd^{2+} are inhibitory at higher concentrations, whereas Ca^{2+} is not.

ATP hydrolysis and ATP synthesis performed by the F_0F_1-ATPase of *Rhodospirillum rubrum* are dependent on the presence of divalent cations. Mg^{2+} is generally regarded as the physiological one. However, other divalent cations have been shown to promote ATP hydrolysis or synthesis (Nishimura, 1962; Bose & Gest, 1965; Johansson et al., 1971). ATP hydrolysis by *R. rubrum* chromatophores in the presence of Mg^{2+} is stimulated by addition of uncouplers (Baltscheffsky, 1964; Horio et al., 1965; Fisher & Guillory, 1967; Horiuti et al., 1968; Edwards & Jackson, 1976). Furthermore, Mg-ATP hydrolysis has been shown to induce proton pumping in *Rhodobacter capsulatus* chromatophores (Melandri et al., 1972).

Solubilized and purified *R. rubrum* F_1-ATPase has lost its ability to hydrolyze ATP in the presence of Mg^{2+}. Instead, the enzyme shows relatively high hydrolytic activity in the presence of Ca-ATP (Johansson et al., 1973). However, unmasking of the Mg^{2+}-ATPase activity and inhibition of the Ca^{2+}-ATPase activity can be accomplished by hydrophobic compounds, such as diethylstilbestrol (Strid et al., 1988), or detergents, e.g., *n*-octyl β-D-glucopyranoside (octyl glucoside),[1] nonanoyl-*N*-methylglucamide, and lauryldimethylamine oxide (Norling et al., 1988).

Recently, a paper occurred dealing with the hydrolysis of ATP by the F_0F_1-ATPase of chloroplasts when Ca^{2+} was used as the divalent cation (Pick & Weiss, 1988). This activity had an absolute requirement for a protonmotive force and was inhibited by uncouplers. No induction of ΔpH or $\Delta\psi$ was found during hydrolysis of Ca-ATP. An intrinsic uncoupling

† This work was partly supported by grants from the Natural Science Research Council, Sweden, to P.N. and to Prof. Margareta Baltscheffsky

[1] Abbreviations: BChl, bacteriochlorophyll; DES, diethylstilbestrol; FCCP, carbonyl cyanide *p*-(trifluoromethoxy)phenylhydrazone; octyl glucoside, *n*-octyl β-D-glucopyranoside.

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of the CF_0F_1-ATPase was proposed (Pick & Weiss, 1988).

The activities of the F_0F_1-ATPase of *R. rubrum* are examined in this paper, with different divalent cations present to test the hypothesis of intrinsic uncoupling and also to find out which divalent cations would possibly induce such an effect.

Experimental Procedures

Preparation of Chromatophores. Chromatophores were prepared from light-grown cells of *Rhodospirillum rubrum* strain S1 (Baltscheffsky, 1967; Strid et al., 1987), and the bacteriochlorophyll (BChl) concentration was estimated (Clayton, 1963) according to standard methods.

Preparation of Purified F_1-ATPase. Purification of F_1-ATPase from *Rhodospirillum rubrum* chromatophores was conducted according to a protocol described previously (Norling et al., 1988).

ATP Hydrolysis. The ATP hydrolysis was determined by a colorimetric assay of the released P_i (Rathbun & Betlach, 1969; Nyrén et al., 1986). The experiments were carried out for 15 or 30 min as indicated below and in the presence of 0.8 μM BChl or 3 μg of F_1 protein. The concentrations of the divalent cations used were the concentrations found to give the highest ATP hydrolysis rates with 2.5 mM ATP (Figure 1). The salts used were the chlorides ($M-Cl_2$ where M represents metal) except for Zn where the acetate was the choice, for Ba^{2+} where $Ba(OH)_2$ was used, and for Pb^{2+} which was present as $Pb(NO_3)_2$. There were no differences in hydrolysis rate between experiments with $MgCl_2$ and magnesium acetate.

ATP-Induced Proton Pumping. To measure qualitatively the proton pumping induced by ATP or light, the quenching of the fluorescence of 9-amino-6-chloro-2-methoxyacridine (ACMA) was studied. The probe is sensitive to the pH gradient in a vesicle system, and its fluorescence (excitation, 415 nm; emission, 485 nm) is quenched when a ΔpH is built up. The membrane potential of the chromatophores was converted to a proton gradient by including 1 μM valinomycin and 50 mM KCl in the assay medium which also contained 80 mM glycylglycine–NaOH, pH 7.4, 0.2 mM sodium succinate, 0.8 μM BChl, 0.25 mM ATP, and 0.4 μM 9-amino-6-chloro-2-methoxyacridine. The concentration of ATP was only 0.25 mM, since higher concentrations severely quenched the fluorescence. Hence, the concentrations of the divalent cations were also decreased with a factor of 10 in relation to those used in the ATPase assays.

ATP Synthesis. By use of the luciferin/luciferase technique, picomoles of ATP can be detected. However, Mg^{2+} (5 mM) is present in commercial assay kits. Obviously, continuous monitoring of ATP by using different cations cannot be measured in this way. To circumvent this problem, ATP synthesis was first performed in a medium containing 0.1 M glycylglycine–NaOH, pH 7.4, 0.5 mM ADP, 1 mM P_i, 0.9 mM sodium succinate, 0.8 μM BChl, and divalent cations at concentrations given below. The samples were put in a water bath and illuminated. FCCP was included or omitted as shown below. The reaction was started by the addition of chromatophores or ADP, the latter in the sets of experiments where FCCP was used. After 10 min, the reaction was terminated by the addition of trichloroacetic acid to a final concentration of 4.8% (w/v). An aliquot (1 μL) of the sample was added to the assay medium in the luminometer. The 1-mL assay medium consisted of 0.95 mL of 0.1 M glycylglycine–NaOH, pH 7.75, 2 mM EDTA, and 50 μL of ATP-monitoring reagent (from LKB-Wallac, Turku, Finland). Standard amounts of ATP were added before and after the addition of the sample aliquot. The concentrations of divalent cations were the same as in the ATP hydrolysis experiments.

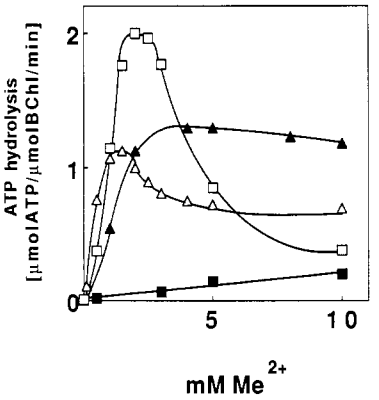

FIGURE 1: Rate of ATP hydrolysis by *R. rubrum* chromatophores as a function of the concentrations of various divalent cations. The assay medium contained 0.1 M glycylglycine–NaOH, pH 7.4, 2.5 mM ATP, 0.8 μM BChl, and Mg^{2+} (△), Ca^{2+} (▲), Cd^{2+} (□), or Ni^{2+} (■).

Results

Hydrolysis of M-ATP by R. rubrum Chromatophores. Edwards and Jackson (1976) showed that optimal concentrations of Mg^{2+} and Ca^{2+} exist for the hydrolysis of ATP by *R. rubrum* chromatophores. The optimum for Mg^{2+} was later shown to be dependent not simply on the concentration of Mg^{2+} but also on the ratio Mg/ATP (Oren & Gromet-Elhanan, 1979). However, the optimum for Ca^{2+} could not be reproduced (Lücke & Klemme, 1976; Oren & Gromet-Elhanan, 1979). It is not known whether or not optimal concentrations occur for other divalent cations.

Figure 1 shows the rates of ATP hydrolysis as a function of the concentration of the divalent cations Mg^{2+}, Ca^{2+}, Cd^{2+}, and Ni^{2+}. The concentration of ATP in these experiments was 2.5 mM. At 1.5 mM Mg^{2+}, the optimum was found. A similar optimum was found at 2.0 mM for Cd^{2+}, as well as for Co^{2+} and Mn^{2+}, and at 2.5 mM for Zn^{2+} (the three latter curves are not shown). For Ca^{2+}, a plateau was found at 4 mM and above, whereas the very low activity in the presence of Ni^{2+} continued to increase up to at least 10 mM. The optimal concentrations seem to be somewhat dependent on the chromatophore preparation: the optimum for Mg^{2+} was found to vary between 1 and 2 mM. In the following experiments (except in the case of proton pumping; see below), these optimal concentrations were used for Mg^{2+}, Cd^{2+}, Co^{2+}, and Zn^{2+}. For Ca^{2+}, the concentration used was 5 mM, and for Ni^{2+}, 10 mM was the choice.

It is generally accepted that high concentrations of Mg^{2+} inhibit ATP hydrolysis (Oren & Gromet-Elhanan, 1979; Norling et al., 1988). It is also obvious from the results above that not only free Mg^{2+} is inhibitory at higher concentrations but also Co^{2+}, Mn^{2+}, and Zn^{2+}. Ca^{2+}, on the other hand, is not inhibitory at higher concentrations. Thus, the results of Lücke and Klemme (1976) and of Oren and Gromet-Elhanan (1979) are strengthened in this respect. Furthermore, Figure 2 shows the effect of $[Mg^{2+}]$ on the Ca-ATP hydrolysis and the effect of $[Ca^{2+}]$ on the Mg-ATP hydrolysis. Clearly, low concentrations of Mg^{2+} are inhibitory of the Ca-ATP hydrolysis, with 50% inhibition at 0.2 mM Mg^{2+} (see insert of Figure 2), whereas Mg-ATP hydrolysis is less sensitive to competition from Ca^{2+}; 40% inhibition of Mg-ATP hydrolysis is attained at 8 mM Ca^{2+}.

The hydrolysis of ATP with Mg^{2+} as the divalent cation is stimulated by additions of uncouplers (Baltscheffsky, 1964; Horio et al., 1965; Fisher & Guillory, 1967; Horiuti et al., 1968; Edwards & Jackson, 1976), whereas the situation is unclear when Ca^{2+} is present (Johansson et al., 1971; Edwards & Jackson, 1976; Strid et al., 1988). A reexamination of

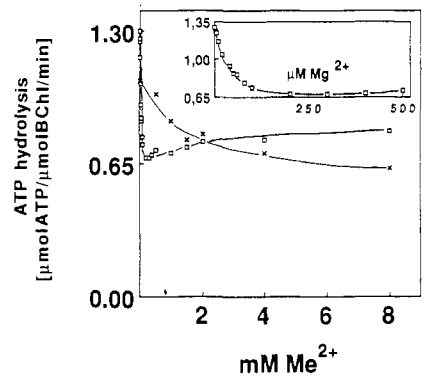

FIGURE 2: Titration of the Mg-ATPase activity with Ca^{2+} and the Ca-ATPase activity with Mg^{2+}. The reaction medium consisted of 0.1 M glycylglycine–NaOH, pH 7.4, 2.5 mM ATP, 0.8 μM BChl, (×) 1.5 mM $MgCl_2$, and (□) 5 mM $CaCl_2$. The reaction was started by addition of ATP and terminated with trichloroacetic acid after 30 min. The insert shows an enlargement of the curve for the titration of the Ca-ATPase with Mg^{2+}.

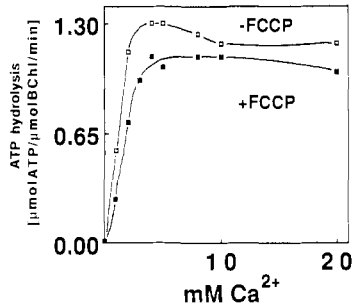

FIGURE 3: Hydrolysis of ATP vs the concentration of Ca^{2+}. The medium consisted of 0.1 M glycylglycine–NaOH, pH 7.4, 2.5 mM ATP, 0.8 μM BChl, and (■) 0.5 μM carbonyl cyanide *p*-(trifluoromethoxy)phenylhydrazone. The reaction was started by addition of ATP and terminated with trichloroacetic acid after 15 min.

uncoupler effects on Ca-ATP hydrolysis reveals that no stimulation occurs (Figure 3). Instead, a slight inhibition is obtained. This inactivation was explained by an inhibitory effect of ethanol, the solvent in which the FCCP is present, at low concentrations (Strid, 1989) and is not the effect of any deactivation of the enzyme by FCCP itself. As shown in Table I, hydrolysis of ATP in the presence of Cd^{2+}, Co^{2+}, Mn^{2+}, or Zn^{2+} is stimulated by FCCP.

Table I also shows the effect of oligomycin on hydrolysis of ATP in the presence of different cations. Ca-ATP hydrolysis is much less sensitive to this drug than is ATP hydrolysis in the presence of any of the other divalent cations: 1.3 μM oligomycin inhibited to 28% the hydrolytic activity in the presence of Ca^{2+} but inhibited more strongly the Cd-ATPase (60%), the Co-ATPase (75%), the Mg-ATPase (82%), the Mn-ATPase (75%), and the Zn-ATPase (69%). Also, previously the Ca-ATP hydrolysis of *R. rubrum* chromatophores has been shown to be less sensitive to oligomycin than the Mg-ATP hydrolysis, 64% and 80% inhibition, respectively (Johansson et al., 1971). However, although the concentrations of oligomycin were approximately 14 μM (Johansson et al., 1971) in their experiments (10-fold higher concentrations than we used in this study), the inhibition of the Mg-ATPase was the same as we obtained. Thus, the concentrations used by Johansson et al. (1971) were too large to elucidate efficiently the less severe inhibition of the Ca-ATPase by oligomycin.

ATP-Induced Proton Pumping by R. rubrum Chromatophores. Figure 4 shows typical traces of three experiments performed in the presence of the fluorescent probe 9-amino-6-chloro-2-methoxyacridine to elucidate the qualitative differences in ATP-induced proton pumping when different cations were present. Note that valinomycin and K^+ were present to convert the membrane potential to ΔpH. Illumination of chromatophores or addition of $MgCl_2$ to a sample containing chromatophores and ATP (Figure 4A) gives rise to quenching of the 9-amino-6-chloro-2-methoxyacridine fluorescence, which indicates the buildup of a proton gradient. This gradient is dissipated by FCCP. In Figure 4B is shown the corresponding trace where $CaCl_2$ was used instead of $MgCl_2$, and no proton pumping was observed. It is also obvious that this addition of Ca^{2+} in no way inhibits proton pumping induced either by light or by ATP with Mg^{2+} as the divalent cation; i.e., no uncoupling of the chromatophore membranes by Ca^{2+} occurs. Figure 4C shows the control trace where FCCP is added prior to ATP and cations. Note that the decrease of fluorescence upon addition of ATP and the increase of fluorescence induced by $CaCl_2$ (Figure 4A,B) also are found under uncoupled conditions in Figure 4C.

Table II shows the qualitative results from the studies of the quenching of 9-amino-6-chloro-2-methoxyacridine by light and by ATP together with the different cations. Light and

Table I: Results of the Assay of the Hydrolytic Activity of *R. rubrum* Chromatophores[a]

cation	hydrolysis rate [μmol of ATP (μmol of BChl)$^{-1}$ min^{-1}]		
	control	+2 μM FCCP	+1.3 μM oligomycin
1.5 mM Mg^{2+}	1.1	3.3	0.2
5.0 mM Ca^{2+}	1.4	1.0	1.0
2.0 mM Cd^{2+}	2.0	2.5	0.8
2.0 mM Co^{2+}	0.8	1.8	0.2
2.0 mM Mn^{2+}	1.2	2.9	0.3
10 mM Ni^{2+}	0.2	0.1	0.06
2.5 mM Zn^{2+}	1.6	4.4	0.5
2.0 mM Ba^{2+}	0	0	nd
2.0 mM Sr^{2+}	0	0	nd

[a] The assay medium contained 0.1 M glycylglycine–NaOH, pH 7.4, 2.5 mM ATP, 0.8 BChl, and additions as indicated. nd, not determined.

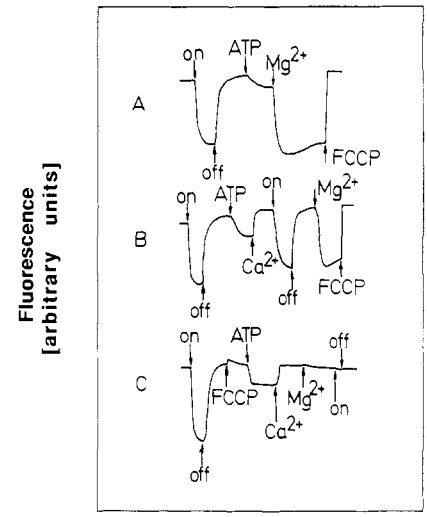

FIGURE 4: Traces from 9-amino-6-chloro-2-methoxyacridine studies of ATP- and light-induced proton pumping. The assay medium contained 80 mM glycylglycine–NaOH, pH 7.4, 50 mM KCl, 0.2 mM sodium succinate, 0.8 μM BChl, 0.4 μM 9-amino-6-chloro-2-methoxyacridine, and 1 μM valinomycin. 0.25 mM ATP, 0.15 mM $MgCl_2$, 0.5 mM $CaCl_2$, and 0.25 μM carbonyl cyanide *p*-(trifluoromethoxy)phenylhydrazone were added, and light was switched on or off where indicated by arrows.

Table II: Results of Qualitative Measurements of 9-Amino-6-chloro-2-methoxyacridine Quenching[a]

source of energy	quenching
light	++
Ca-ATP (0.5 mM Ca^{2+})	-
Cd-ATP (0.2 mM Cd^{2+})	+
Co-ATP (0.2 mM Co^{2+})	++
Mg-ATP (0.15 mM Mg^{2+})	++
Mn-ATP (0.2 mM Mn^{2+})	++
Zn-ATP (0.25 mM Zn^{2+})	+
Ba-ATP (0.2 mM Ba^{2+})	-
Sr-ATP (0.2 mM Sr^{2+})	-
M-ATP + FCCP	-

[a] Quenching is indicated by (+), strong quenching by (++), and no quenching by (-). The medium consisted of 80 mM glycylglycinehNaOH, pH 7.4, 50 mM KCl, 0.4 μM 9-amino-6-chloro-2-methoxyacridine, 2.5 μM valinomycin, 0.2 mM sodium succinate, 0.8 μM BChl, 0.25 mM ATP, and additions as indicated.

Table III: Results of the Assay of the Rate of Synthesis of ATP of *R. rubrum chromatophores*[a]

cation	synthesis rate [μmol of ATP (μmol of BChl)$^{-1}$ min^{-1}]
discontinuous	
1.5 mM Mg^{2+}	7.4
1.5 mM Mg^{2+} + 2 μM FCCP	1.2
5.0 mM Ca^{2+}	0.2
5.0 mM Ca^{2+} + 2 μM FCCP	0
2.0 mM Cd^{2+}	1.1
2.0 mM Co^{2+}	6.1
2.0 mM Mn^{2+}	4.9
10 mM Ni^{2+}	3.1
2.5 mM Zn^{2+}	3.9
2.0 mM Ba^{2+}	1.0
2.0 mM Sr^{2+}	1.0
50 mM K^+	1.8
no divalent cation added[b]	1.8
no added divalent cation[b] + 2 μM FCCP	0
continuous	
5 mM Mg^{2+}	8.4

[a] The assay medium contained 0.1 M glycylglycine–NaOH, pH 7.4, 0.9 mM sodium succinate, 0.5 mM ADP, 1 mM NaP_i, 0.8 μM BChl, and additions as indicated. [b] The ions present are Na^+ ions from the adjustment of the buffer, and the Na^+ counterions of the ADP, the succinate, and the P_i.

Mg-ATP (see also Figure 4) as well as Co-ATP and Mn-ATP seem to be equally good as inducers of a proton gradient. The quenching of the fluorescence in the presence of Cd-ATP and Zn-ATP was somewhat less than in the presence of the other ions.

ATP Synthesis by R. rubrum Chromatophores. ATP is synthesized from ADP and P_i with light as the source of energy. The amount of ATP produced was measured by the luciferin/luciferase technique (Table III). In the presence of Mg^{2+}, the average synthesis rate was 7.4 μmol of ATP·(μmol of BChl)$^{-1}$·min^{-1} as measured by the discontinuous method, whereas continuous monitoring gave an initial rate of 8.4 μmol of ATP·(μmol of BChl)$^{-1}$·min^{-1}. When measured by the discontinuous method, no significant difference in synthesis rate was observed between 0.5 and 2 mM Mg^{2+}, whereas the rate was approximately half between 5 and 50 mM Mg^{2+} (not shown). Only 16% of the amount of ATP synthesized in the absence of uncouplers was formed after addition of 2 μM FCCP (Table III). If Ca^{2+} is used instead of Mg^{2+}, even less ATP is formed: approximately 3% of the amount of ATP formed in the presence of Mg^{2+}.

Obviously, Co^{2+}, Mn^{2+}, and Zn^{2+} were also able to promote ATP synthesis in *R. rubrum* chromatophores (Table III), although we found lower rates than in the presence of Mg^{2+}. The rate for Cd^{2+} was not distinguishable from the background.

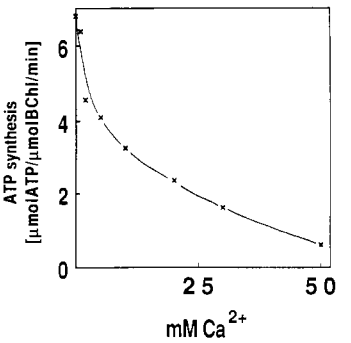

FIGURE 5: Titration of Mg-dependent ATP synthesis with Ca^{2+}. The ATP synthesis was performed in 0.1 M glycylglycine–NaOH, pH 7.4, 0.5 mM ADP, 1 mM P_i, 0.9 mM sodium succinate, 0.8 μM BChl, 1.5 mM $MgCl_2$, and $CaCl_2$ as indicated in the figure. The reaction was stopped after 10 min by addition of trichloroacetic acid. One microliter of the sample was injected into a luminometer containing the luciferin/luciferase assay medium described above. BChl stands for bacteriochlorophyll.

Table IV: Results of the Assay of the Hydrolytic Activity of Purified *R. rubrum* F_1-ATPase[a]

	hydrolysis rate [μmol of ATP (μmol of BChl)$^{-1}$ min^{-1}]			
		+50 mM HCO_3^-		
cation	no addition		+20 μM DES	+20 mM OG
1.5 mM Mg^{2+}	0	0.12	0.79	1.2
5.0 mM Ca^{2+}	2.2	2.4	0.36	0.17
2.0 mM Cd^{2+}	0.72	0.54[b]	0.15[b]	0.33[b]
2.0 mM Co^{2+}	0	0.13	0.94	0.26
2.0 mM Mn^{2+}	0	1.4	1.6	0.69
10 mM Ni^{2+}	0	>0	0.05	0.49
2.5 mM Zn^{2+}	0.03	0.12[b]	0.30[b]	0.39[b]
5.0 mM Sr^{2+}	0	0	0	0
5.0 mM Ba^{2+}	0	0[b]	0[b]	0[b]

[a] The assay medium consisted of 50 mM Tris-HCl, pH 7.5, 3 μg of protein, 2.5 mM ATP, and additions as indicated. [b] In the presence of HCO_3^-, some precipitation occurred which presumably lowered the activity.

Ca^{2+} inhibits ATP synthesis in the presence of 1.5 mM Mg^{2+}. A titration curve is shown in Figure 5. It is similar to the pattern of inhibition by Ca^{2+} of the Mg-ATP hydrolysis. We obtain a 45–50% inhibition at 8 mM Ca^{2+} and a 90% inhibition at 50 mM Ca^{2+}.

ATP Hydrolysis by Purified F_1-ATPase from R. rubrum. In Table IV are given the results of the experiments on ATP hydrolysis by purified F_1-ATPase. Only Ca^{2+} and Cd^{2+} gave substantial rates in the absence of activators. The rate in the presence of Ca^{2+} was 3-fold higher than in the presence of Cd^{2+}. By including HCO_3^- (Webster et al., 1977), a Mg-ATPase activity was unmasked. In the same way, F_1 exhibited Co-ATP, Mn-ATP, and Zn-ATP hydrolysis in the presence of HCO_3^-. Hydrophobic agents such as DES (Strid et al., 1988) and octyl glucoside (Norling et al., 1988) have been shown to stimulate the Mg-ATPase activity of purified F_1-ATPase further but also to inhibit the Ca-ATPase activity of the same enzyme preparation. The ATPase activity in the presence of Co^{2+}, Mn^{2+}, and Zn^{2+} was stimulated by DES, whereas in the presence of Cd^{2+} the ATPase was inhibited by DES. In the same way, the Co-ATP and Zn-ATP hydrolyses were increased by octyl glucoside, and Cd-ATP hydrolysis decreased. However, the pattern was not followed by the Mn-ATPase which was inhibited by octyl glucoside. Note that

precipitation of the hydrocarbonates of Cd and Zn occurred. This affects the quantitativity of the measurements, whereas the results should be qualitatively applicable.

Other Divalent Cations Tested. Two larger alkaline earths, Sr^{2+} and Ba^{2+}, were also tested. The hydrolytic (Table I) and proton pumping (Table II) activities were very near zero in the presence of both these ions, and the ATP synthesis activity was less than the background (Table III). No activity of purified F_1-ATPase was obtained either (Table IV). These ions are probably too large to be able to promote the reactions of the ATP synthase.

Ni^{2+} is another ion which was included in the experiments. It is obvious from Figure 1 that the Ni-ATPase did not have any optimum below 10 mM and showed a low activity, at its best approximately 20% of the Mg-ATPase activity (Table I). Although no stimulation by FCCP was observed, the hydrolysis was inhibited by oligomycin (Table I). The decrease in 9-amino-6-chloro-2-methoxyacridine fluorescence in the presence of Ni^{2+} and ATP was very low (not shown). The ATP synthesis rate seemed to be distinctly higher than the background (Table III), and the ATPase activity of purified F_1 also showed a low but definite rate which was stimulated by DES and octyl glucoside.

Other ions that were used are Fe^{2+}, Sn^{2+}, Hg^{2+}, Pb^{2+}, and Cu^{2+}. These either did not promote any activities or were oxidized, precipitated, or reacted with the buffer.

Discussion

Two Groups of Divalent Cations. The divalent cations which promote the reactions catalyzed by the ATP synthetase of *R. rubrum* can be divided into two major groups. The "Mg^{2+}-group" is characterized by the following features: (i) uncoupler-stimulated ATPase in chromatophores that has an optimum in its demand for divalent cations (this hydrolysis is oligomycin sensitive); (ii) ATP-induced proton pumping; (iii) light-induced ATP synthesis in chromatophores; (iv) no, or very low, ATPase activity in purified F_1-ATPase, unmasked by hydrophobic compounds such as DES and octyl glucoside. The characteristics for the "Ca^{2+}-group" are (i) no stimulation by FCCP of the ATP hydrolysis in chromatophores; the activity shows only low sensitivity to oligomycin; (ii) no proton gradient is built-up by M-ATP; (iii) no light-induced ATP synthesis is accomplished; (iv) a high hydrolytic activity occurs in purified F_1-ATPase which is decreased by the hydrophobic compounds tested.

Mg^{2+}, Co^{2+}, Mn^{2+}, and Zn^{2+} are suggested to be members of the former group due to the results described above. Ca^{2+} is a member of the second group, and Cd^{2+} falls somewhere in between the groups. Thus, in the presence of ions of the "Mg^{2+}-group", the classical pattern of ATP hydrolysis and ATP synthesis coupled to the protonmotive force is exhibited.

Intrinsic Uncoupling by Ca^{2+}. There exists a calcium–proton antiport in *R. rubrum* chromatophores (Davidson & Knaff, 1981). The import of Ca^{2+} is driven by an efflux of H^+. This means that the proton gradient is dissipated when Ca^{2+} is transported into the chromatophores. The lack of photophosphorylation in the presence of Ca^{2+} and the lack of appearance of a ΔpH across the chromatophore membrane when Ca-ATP is hydrolyzed could possibly be explained by an antiport of this kind. However, if the antiport were to dissipate the protonmotive force, no buildup of a ΔpH would occur during illumination or during Mg-ATP hydrolysis when Ca^{2+} is present. Since such induction of ΔpH does occur, no general uncoupling of the membrane or any calcium–proton antiport can account for the lack of photophosphorylation and buildup of a proton gradient with Ca-ATP.

Instead, our results support the proposal of Pick and Weiss (1988) that intrinsic uncoupling occurs in the F_0F_1-ATPase when Ca^{2+} is used as cation. Their experiments were carried out in chloroplasts. However, they do not discuss any possible physiological role of this finding.

The intrinsic uncoupling is likely to be caused by a conformational change in the enzyme governed by the size of the divalent cations. The Ca^{2+} ion has a diameter of 0.95 Å and Cd^{2+} 0.92 Å (Hägg, 1979) whereas the members of the "Mg^{2+}-group" all have smaller ion diameters (Mg^{2+}, 0.65 Å; Zn^{2+}, 0.70 Å; Co^{2+}, 0.72 Å; Mn^{2+}, 0.80 Å; Hägg, 1979). Sr^{2+} (1.10 Å) and Ba^{2+} (1.30 Å) are probably too large to be able to bind to the active site of the enzyme. It should be emphasized that the dissociation constants (K_d) for the M-ATP complexes are all of the same order of magnitude (Sillén & Martell, 1964).

Significance of the Intrinsic Uncoupling. Because of the high Ca^{2+} concentration used in these in vitro studies, the physiological significance of the data presented is not apparent. However, Mg^{2+} and Ca^{2+} are both needed for cells of *Rhodospirillum rubrum* to grow. In this case, 0.8 mM Mg^{2+} and 0.5 mM Ca^{2+} were used in the growth medium.

Whatever the physiological role of this intrinsic uncoupling, it would be disastrous to the cell if it was not kept under strict control, i.e., the Mg^{2+}:Ca^{2+} ratio kept high by reducing the concentration of Ca^{2+} by complexation, by precipitation in the form of granules of Ca salts, or by extrusion by active transport. However, the Ca^{2+} must be mobilizable when needed in different physiological processes. For the future understanding of the uncoupling mechanism of the F_0F_1-ATPase, the determination of intracellular Mg^{2+} and Ca^{2+} concentrations is very important.

Regulation by Divalent Cations. Six divalent cation binding sites have been suggested to reside on each molecule of F_1 from beef heart mitochondria. One of the sites is supposed to be crucial for structure and to obligatory bind Mg^{2+}, whereas another is assumed to be a regulatory site (Dagget et al., 1985). Ca^{2+} does not bind to either of these two sites, and neither site is thought to bind the metal of the M^{2+}–nucleotide complexes.

Among other interesting findings in these experiments is the observation that the Ca-ATPase is inhibited by Mg^{2+} and vice versa, the former being much more sensitive (Figure 2). This is not a result of different K_ms for Mg- and Ca-ATP (Müller et al., 1979). Instead, the results imply that Ca^{2+} does not act at any regulatory divalent cation binding sites. This is supported by the results of Figures 1 and 3. In the former figure, an optimum for the concentrations of the divalent cations of the "Mg^{2+}-group" was obtained, once more indicating the regulatory role of free Mg^{2+}, whereas the Ca-ATPase activity is not controlled in that way. This observation puts more emphasis on the conclusion that the Mg binding regulatory sites are not susceptible to the binding or the action of Ca^{2+}.

Purified F_1-ATPase and the Divalent Cations. In addition to the differences between the influence of the ions of the "Mg^{2+}-group" and Ca^{2+} on the F_0F_1-ATPase that have been investigated in this paper, the hydrolytic activity of purified F_1-ATPase from *Rhodospirillum rubrum* is affected differently by hydrophobic compounds if ions of the "Mg^{2+}-group" are used instead of Ca^{2+}. Untreated purified F_1-ATPase shows only hydrolytic activity when Ca^{2+} and Cd^{2+} are present. However, when DES or octyl glucoside is added, an ATPase activity in the presence of ions from the "Mg^{2+}-group" is unmasked, whereas the Ca-ATPase is inhibited.

In the purified F_1-ATPase, an important conformational change is likely to occur as a result of dissociation from the membrane. This appears as an extremely low hydrolytic activity in the presence of Mg^{2+}, whereas Ca^{2+} promotes ATP hydrolysis. Clearly, a larger ion than any of those in the Mg^{2+} group is needed to present the M–ATP complex in a correct fashion at the active site. However, the addition of DES or octyl glucoside makes the enzyme susceptible to ATP complexed to an ion of the Mg^{2+} group. The K_m remains the same whereas the V_{max} is increased (Norling et al., 1988). This might imply that a hydrophobic interaction between the F_1 part and the F_0 and/or the membrane itself exists in vivo. However, the state of the enzyme in the presence of DES and octyl glucoside cannot be identical with the native state, since the Ca-ATPase activity of chromatophores and solubilized and reconstituted F_0F_1-ATPase is inhibited by DES (Strid et al., 1988). Possibly, the hydrophobic compounds also interact elsewhere on F_1. The molecules may bind in hydrophobic crevices on the surface of F_1, perhaps even at or near the active site where hydrophobic amino acid residues are thought to be located (see below).

Possible Mechanisms. There are mainly two different theories for the coupling of the "energy-rich" protons translocated by F_0 to the net synthesis of ATP. In the first concept, the protons are considered to be conducted to the catalytic site where they are directly involved in ATP formation (Mitchell, 1974, 1985). The second hypothesis suggests that the protons passing through F_0 induce a conformational change in the enzyme that leads to release of ATP (Boyer, 1975; Harris, 1978; Jencks, 1980).

A possible mechanism of "intrinsic uncoupling" in the membrane-bound state of the enzyme could be as follows if a conformational transfer of energy of the translocated protons is assumed: The binding of a Ca–nucleotide complex (which would be larger than a Mg–nucleotide complex) by the enzyme fails to induce the conformational change required to facilitate proton translocation through F_0.

If the proton to be translocated is thought either to participate in catalysis or to pass through the active site, another hypothesis could be the following: On binding the large Ca–nucleotide complex to the active site, glycine-rich and aspartate-containing strands might fail to keep water out of the binding site, and the proton could escape into the bulk phase instead of being translocated.

Since the F_1-ATPase alters its metal requirement (and probably its conformation) when solubilized from the membrane, it would be plausible to suggest that the large Ca–ATP complex is the only M^{2+}–nucleotide that binds in a manner that is favorable for catalysis to take place [Ca^{2+} has been shown to be more flexible than Mg^{2+} in accepting ligands; see Williams (1976, 1980)]. Added hydrophobic compounds might interact with hydrophobic domains and "tighten" the binding site. Then, the smaller M^{2+}–ATP complexes would be presented in a way that allows hydrolysis, whereas Ca–ATP has become too large.

Concluding Remarks. The answer to the intriguing question of what the mechanism might be behind the differences between the "Mg^{2+}-group" ions and Ca^{2+} in relation to the F_0F_1-ATPase lies in the future. So does the understanding of any possible physiological role for this difference. However, our present results certainly imply that the concentration of free Ca^{2+} or the ratio [free Mg^{2+}]/[free Ca^{2+}] has to be kept under strict control not to waste the precious ATP into pure entropy.

Acknowledgments

9-Amino-6-chloro-2-methoxyacridine was a gift from Prof. Jan Rydström and Dr. Bengt Persson, who are greatly acknowledged. We also thank Elisabeth Welin and Kaj Grandien for performing some of the preliminary experiments on the ATP hydrolysis, Karl Axel Strid for helping us out with the language, and Prof. Margareta Baltscheffsky in whose laboratory this work was performed.

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Binding of Sulfonamide and Acetamide to the Active-Site Zn^{2+} in Carbonic Anhydrase: A Theoretical Study†

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ABSTRACT: Self-consistent field molecular orbital (SCF MO) calculations at both 4-31G and STO-3G levels have been used to examine the binding conformations of sulfonamide and acetamide compounds to the active site of carbonic anhydrase. The results are as follows: (1) sulfonamide binds to the Zn^{2+} ion in its deprotonated form through the sulfonamide nitrogen to the fourth coordination site of the metal ion; (2) acetamide as neutral species binds to the basic form of the enzyme through the carbonyl oxygen to the fifth coordination site of the metal ion; and (3) the acetamidate ion binds to the acid form of the enzyme through the amide nitrogen to form a tetracoordinated metal complex with three histidine ligands. Analysis of the effects of individual active-site residues on the binding conformations of these inhibitors suggests that metal alone favors bidentate coordination of sulfonamidate and acetamidate complexes and that electron donation from three histidine ligands to the metal ion determines the formation of a tetracoordinated metal complex, which is further stabilized by the presence of Thr 199, as it receives one hydrogen bond from the sulfonamide NH^- or from the acetamide NH^- and donates a backbone NH hydrogen bond to a sulfonamide oxygen. The calculated binding conformation of sulfonamide and the hydrogen-bonding interactions between sulfonamide and the enzyme are consistent with the X-ray diffraction study of the AMSulf–HCA II complex. However, no X-ray structures are available for amide–HCA II complexes. Finally, a three-step binding mechanism is proposed to explain the experimentally observed slow association kinetics of amide compounds: (1) initial binding of an amide compound through the carbonyl oxygen to the fifth coordination site of the metal ion; (2) proton transfer from the amide nitrogen to the metal-bound OH^-; and (3) release of a metal-bound water molecule and subsequent coordination of the amidate compound through the amide nitrogen to the metal ion. The proton-transfer process of step 2 is considered to be rate limiting.

Carbonic anhydrase is a zinc metalloenzyme that catalyzes the reversible hydration of CO_2 to bicarbonate ion and a proton. In human carbonic anhydrase II (HCA II) the maximal turnover number is 10^6 s^{-1} at 25 °C. It is now widely accepted that in HCA II (Lindskog, 1983; Lindskog et al., 1984; Lipscomb, 1983; Pocker & Sarkanen, 1978; Prince, 1979; Coleman, 1980; Silverman & Lindskog, 1988) the initial nucleophilic attack occurs by a Zn^{2+}-bound hydroxide ion and that the subsequent proton transfer is catalyzed by a non-Zn^{2+}-liganded histidine or another proton-transfer group[1] (Forsman et al., 1988) and by buffer in HCA II. A plausible catalytic mechanism for the hydration of CO_2 is given in Figure 1.

It is known that tightly bound inhibitors of carbonic anhydrase are mononegative anions (e.g., halides, N_3^-, NCO^-, NCS^-, CN^-) or neutral molecules (e.g., sulfonamide) with low pK_a values that can deprotonate to form anionic species during metal binding (Pocker & Sarkanen, 1978). Other neutral inhibitors, such as alcohols, organic solvents, and amide compounds (Verpoorte et al., 1967; Pocker & Stone, 1968; Whitney et al., 1967; Whitney, 1970, 1973; Pocker & Sarkanen, 1978; Bertini & Luchinat, 1983), have high pK_a values and are in general weak inhibitors.

Sulfonamide compounds, with a pK_a of about 10 for an unbound benzenesulfonamide (Taylor & Burgen, 1971; Taylor et al., 1970, 1971; Harrington & Wilkins, 1980; King & Burgen, 1976), are known to bind specifically (K_i = 0.01–1 μM) (Kanamori & Roberts, 1983) to the zinc ion using a deprotonated form of the NH_2 group. The observed large ^{111}Cd–^{15}N spin-coupling constants between ^{15}N of sulfonamide and ^{111}Cd of ^{111}Cd-substituted HCA indicates a direct coordination of the deprotonated sulfonamidate ion through nitrogen to the Cd ion (Edelhoch et al., 1981; Blackburn et al., 1985). The early X-ray structures of acetazolamide (Diamox)–HCA I (Kannan et al., 1977; Kannan, 1979) and benzenesulfonamide–HCA I (Kannan, 1979) complexes indicated that sulfonamide binds in a bidentate conformation to the metal ion, with the sulfonamide nitrogen replacing the solvent water molecule at the fourth coordination site and one sulfonamide oxygen bound to the fifth coordination site of the metal ion. (For example, Zn–O = 2.7 Å and Zn–N = 2.8 Å in the X-ray structure of the acetazolamide–HCA I complex.) However, in the recently refined X-ray structures of the acetazolamide–HCA II complex to 3-Å resolution (Eriksson

† For support of this research we thank the National Science Foundation (CHE-85-15347, CHE-88-20590 and PCM-77-11398) and the National Institutes of Health (GM06920).

[1] Mutations of His 64 to Lys, Gln, Glu, or Ala change k_{cat} for CO_2 hydration by factors of 1.5–3.5 as compared to the value for the native enzyme at pH 8 and 25 °C.

0006-2960/89/0428-9724$01.50/0