M. (1986) *Eur. J. Biochem. 161*, 787-792.
Schweitz, H., Stansfeld, C. E., Bidard, J.-N., Fagni, L., & Lazdunski, M. (1989) *FEBS Lett. 250*, 519-522.
Smith, C., Phillips, M., & Miller, C. (1986) *J. Biol. Chem. 261*, 14607-14613.
Stansfeld, C. I., & Feltz, A. (1988) *Neurosci. Lett. 93*, 49-55.
Stansfeld, C. E., Marsh, S. J., Parcej, D. N., Dolly, J. O., & Brown, D. A. (1987) *Neuroscience 23*, 893-902.
Stühmer, W., Stocker, M., Sakmann, B., Seeburg, P., Baumann, A., Grupe, A., & Pongs, O. (1988) *FEBS Lett. 242*, 199-206.
Taylor, J. W., Bidard, J.-N., & Lazdunski, M. (1984) *J. Biol. Chem. 259*, 13957-13967.
Weller, U., Bernhardt, U., Siemen, D., Dreyer, F., Vogel, W., & Habermann, E. (1985) *Naunyn-Schmiedeberg's Arch. Pharmacol. 330*, 77-83.

Calcium-Binding ATPase Inhibitor Protein of Bovine Heart Mitochondria. Role in ATP Synthesis and Effect of Ca^{2+} †

Esther W. Yamada* and Norman J. Huzel
Department of Biochemistry and Molecular Biology, University of Manitoba, Winnipeg, Manitoba, Canada R3E 0W3
Received June 2, 1989; Revised Manuscript Received July 28, 1989

ABSTRACT: Submitochondrial particles (A particles) and phosphorylating electron-transport particles (ETP_H) were prepared from bovine heart mitochondria. The A particles either were supplemented with or were depleted of the mitochondrial calcium-binding ATPase inhibitor protein (CaBI). The CaBI-depleted A particles still retained the Pullman–Monroy ATPase inhibitor protein (PMI), and the other particles all contained both CaBI and PMI. ATP synthase and ATPase activities of the particles were measured in similar reaction mixtures by luminescence of firefly luciferin–luciferase. Succinate was the respiratory substrate, and the adenylate kinase inhibitor P^1,P^5-di(adenosine-5′) pentaphosphate was obligatory. The ATP synthase activity of CaBI-depleted A particles was 30–40% of that of the A and ETP_H particles, and its ATPase activity was 7–8 times greater. Reconstitution of the CaBI-depleted A particles with CaBI restored the original ATP synthase and ATPase activities. ATP synthase activity rose about 1.7-fold when A particles were supplemented with additional CaBI and ATPase activity dropped to 9% of the original. Varying Ca^{2+} levels had little or no effect on the ATP synthase and ATPase activities of the CaBI-depleted A particles. In contrast, ATP synthase activity of the other particles was decreased by as much as 70% at the optimal Ca^{2+} concentration of 1 μM, and the ATPase activity of the A and ETP_H particles rose concomitantly by 7–8-fold. The ATP synthase and ATPase activities of all the particles in 1 μM Ca^{2+} became like those of the CaBI-depleted A particles. These changes were reversible; normal activities were restored as Ca^{2+} concentrations were raised above 1 μM. Thus, CaBI influence both ATP synthesis and hydrolysis, and Ca^{2+} modulates both through CaBI.

The calcium-binding ATPase inhibitor protein (CaBI)[1] of mitochondria of rat skeletal muscle and bovine heart (Yamada et al., 1980) was discovered during the course of studies to determine why the energy-dependent uptake of Ca^{2+} by mitochondria takes preference over oxidative phosphorylation (Rossi & Lehninger, 1964; Vercesi et al., 1978) and results in the stimulation of mitochondrial ATP hydrolysis (Hunter et al., 1976; Bygrave, 1977). It was found that Ca^{2+}, in micromolar concentrations, caused the release of CaBI from submitochondrial particles with a concurrent stimulation of from 5-fold to just over 8-fold in the ATPase activity of the particles (Yamada et al., 1980, 1981). The CaBI-depleted particles (Yamada & Huzel, 1988) still retained some amounts of the ATPase inhibitor protein (PMI) first isolated by Pullman and Monroy (1963) from bovine heart mitochondria. The dimeric form of CaBI was the active inhibitor (Yamada et al., 1981). The molecular weights of the monomeric and dimeric forms and the cleavage of the CaBI dimer by thiol compounds were reminiscent of ATPase inhibitor proteins isolated earlier which were believed to be forms of PMI (Knowles & Penefsky, 1972). Paradoxically, however, PMI isolated by the conventional method of alkaline extraction (Pullman & Monroy, 1963) was not cleaved by thiol compounds (Knowles & Penefsky, 1972). Moreover, unlike CaBI, PMI does not contain cysteine residues (Frangione et al., 1981). Later, PMI and CaBI were both isolated from bovine heart mitochondria and shown to be distinct proteins (Yamada & Huzel, 1988).

Pullman and Monroy (1963) showed that PMI inhibited the hydrolytic activity of the ATP synthase·ATPase complex of submitochondrial particles concomitantly with recoupling of the particles and an increase in P:O ratios. Whether PMI simply conserved ATP or had a direct effect on ATP synthesis was the subject of later research by others [see Schwerzman and Pedersen (1986) and Lippe et al., (1988a,b)]. It has been proposed that PMI must be released or translocated to a new site in order that ATP synthesis can proceed. Yet another proposal was that PMI exists in active and inactive forms (Panchenko & Vinogradov, 1985). Recently, the entrapment

† This work was supported by the Medical Research Council of Canada.
* To whom correspondence should be addressed.

[1] Abbreviations: PMI, Pullman and Monroy mitochondrial ATPase inhibitor protein; CaBI, Ca^{2+}-binding mitochondrial ATPase inhibitor protein; Ap_5A, P^1,P^5-di(adenosine-5′) pentaphosphate; TES, *N*-[tris(hydroxymethyl)methyl]-2-aminoethanesulfonic acid.

0006-2960/89/0428-9714$01.50/0

of nucleotides by PMI in reconstituted ATP synthase·ATPase complexes and the release of about one nucleotide during ATP synthesis were shown (Penin et al., 1988).

Not considered in any of the above studies was CaBI. It was decided to prepare submitochondrial particles that contained from none to excess CaBI in order to determine directly the effect of CaBI on ATP synthetic and hydrolytic rates and the effect of Ca^{2+}. Like PMI (Pullman & Monroy, 1963), CaBI restored ATP synthetic activity to uncoupled submitochondrial particles and reduced ATP hydrolytic activity. Unlike PMI, the activity of CaBI was modulated by Ca^{2+}.

Experimental Procedures

Materials. Cytochrome *c*, ATP, AMP, rotenone, antimycin, and P^1,P^5-di(adenosine-5′) pentaphosphate (Ap_5A) were obtained from Sigma. Luciferin–luciferase reagent (ATP monitoring reagent) for use with an LKB luminometer (Model 1251) was obtained from LKB or Sigma. ADP was obtained from Boehringer-Mannheim. Calcium chloride (Suprapur) was obtained from British Drug Houses. Glass-redistilled, deionized water was used throughout.

Methods. Submitochondrial particles (A particles) were prepared from heavy mitochondria from bovine heart (Fessenden & Racker, 1966). Some particles were depleted of CaBI to give CaBI-depleted A particles.[2] Briefly (Yamada & Huzel, 1988), 18 mg of A particles was incubated in 180 mL of 0.36 M mannitol–50 mM Tris/sulfate buffer (pH 7.7) containing 0.085 nmol of $Ca^{2+}/\mu g$ of protein at 0 °C for 30 min. The suspension was centrifuged at 185000*g* for 10 min (Spinco rotor 70Ti) at 4 °C. The pellet was washed once with buffer containing 1 mM EGTA and once with buffer alone and then was resuspended in 0.25 M sucrose.

A particles were also supplemented with additional CaBI (CaBI-supplemented A particles) by the method used by Harris et al. (1974) to supplement submitochondrial particles with PMI. In our case, the A particles (2.26 mg) were incubated with 0.5 mM Mg–ATP and excess CaBI (2.4 μg) in 0.25 M sucrose–15 mM Tris/TES buffer (pH 6.5) for 20 min at 25 °C. The suspension was then centrifuged at 100000*g* (Spinco rotor 65) for 30 min at 4 °C. The resulting pellet was resuspended in 0.25 M sucrose. Electron transport particles (ETP_H) was prepared according to Green and Ziegler (1967). All particles were stored at −70 °C.

The inhibitory activity of CaBI was measured by the ATPase assay used before (Yamada & Huzel, 1988). A unit of ATPase inhibitor protein activity is defined as the amount that resulted in 50% inhibition of 0.2 unit of ATPase activity under the conditions specified (Yamada & Huzel, 1988; Horstman & Racker, 1970). Specific inhibitor protein activity is given in units per milligram of protein. Protein was determined as before (Yamada & Huzel, 1988).

Rates of ATP synthesis and degradation[3] were measured by the luminescence of firefly luciferase in response to ATP and luciferin. Initially, the method of Lemasters and Hackenbrock (1976) was used; it was not possible to attain saturating concentrations of substrates with this assay due to the sensitivity of our luminometer. In the new assay, the reaction mixture for measurement of ATP synthase activity consisted of 0.25 M sucrose, 8 mM $MgSO_4$, 1 mM EDTA, 5 μM cytochrome *c*, 6.25 μM rotenone, 120 μM Ap_5A, 50 mM Tris/acetate buffer, 20 mM potassium phosphate buffer (pH 7.5), 1.2 mM ADP, and enzyme with or without 5 mM succinate in a final volume of 0.2 mL. The mixture was preincubated for 10 min at 30 °C minus ADP to overcome the lag period of about 40 s (Klein & Vignais, 1983) as well as to activate succinate dehydrogenase activity of the particles (Lippe et al., 1988a). ADP was then added to start the reaction, and incubation was continued for an additional 4 min.

The reaction mixture for measurement of ATPase activity was the same as above except that ADP and cytochrome *c* were omitted. After preincubation for 10 min at 30 °C, the reaction was initiated by the addition of 5 mM ATP; incubation was continued for an additional minute.

The reactions in both assays were terminated by a 20-fold dilution into 0.1 M Tris/acetate buffer (pH 7.75) containing 0.1% Triton X-100 and 2 mM EDTA. The ATP content of samples was then determined by the ATP monitoring reagent (luciferin–luciferase) in 0.01 M Tris/acetate–0.2 mM EDTA buffer (pH 7.75) at 25 °C in the luminometer. Luminescence was initiated by the addition of 10 μL of diluted reaction mixture. Luminescence was standardized by the addition of 0.2 μM ATP to the reaction mixture which lacked substrate. Appropriate controls lacking either enzyme or substrate were included with all assays; all were done in duplicate. Buffer media of both assays were aerated for 10 min just prior to preincubation.

Results

ATP Synthase Activity. The adenylate kinase activity of some preparations of submitochondrial particles can interfere with measurements of ATP synthase activity (Harris et al., 1979). In our case, there was significant activity in the absence of the respiratory substrate succinate. The addition of AMP (Lemasters & Hackenbrock, 1976) resulted in nonlinear rates of reaction. Ap_5A, a specific inhibitor of adenylate kinase (Lienhard & Secemski, 1973), inhibited the activity in the absence of succinate. The effective concentration of Ap_5A was 120 μM, comparable to that used by others (Lippe et al., 1988a). Succinate-induced ATP synthase activity was now a linear function of time for at least 4 min and of protein concentration up to 20 μg. Maximal activity obtained at 1.2 mM ADP as others also reported (Matsuno-Yagi & Hatefi, 1985) with a slight falling off at 1.5 mM. There was a small plateau at 150 μM ADP where the activity was about one-fifth of that at saturating concentrations.

ATPase Activity. Activity was linear for just over 1 min and with protein concentration up to 5 μg. Maximal activity was reached at 4 mM ATP and fell off only at 8 mM. It was considered important to keep the incubation medium as close as possible to that used for measurement of ATP synthase activity for better comparison of the activities. Activities were more consistent when Ap_5A was present in the reaction mixture. Replacement of rotenone by antimycin affected the activity of only the CaBI-depleted A particles (see below).

Inhibitor Protein Content of Submitochondrial Particles. Heat-stable protein fractions were prepared from the particles as described before (Yamada & Huzel, 1988). The proteins of the fractions were separated by electrophoresis (Laemmli, 1970) at an acrylamide concentration of 15%. Purified CaBI and PMI were used as markers. The relative amounts of CaBI and PMI in each fraction were estimated from densitometer tracings of the stained gels (Figure 1). All fractions contained a protein corresponding to PMI. Only the CaBI-depleted A particles lacked CaBI. There were in all samples additional proteins that remained close to the top of each gel.

Inhibitor protein activity of the heat-stable fractions was estimated by the standard assay (Yamada & Huzel, 1988). In some cases, 1 μM Ca^{2+} was included in the preincubation

[2] Also called Ca^{2+}-stripped A particles in previous publications (Yamada et al., 1981; Yamada & Huzel, 1988).

[3] Referred to as ATP synthase and ATPase activities, respectively.

Table I: Inhibitor Protein Content of Submitochondrial Particles of Bovine Heart[a]

sample	protein (mg)	inhibitor protein			
		CaBI (units)	PMI (units)	CaBI/PMI (units/units)	CaBI/PM[b]
A particles	0.45	152.2	91.2	1.67	1.60
ETP_H particles	0.20	43.4	35.8	1.21	1.26
CaBI-supplemented A	0.58	262.7	84.3	3.12	3.06
CaBI-depleted A	0.24	0	ND[c]		

[a] A heat-stable protein fraction (Yamada & Huzel, 1988) was prepared from 40 mg of each type of submitochondrial particle. Inhibitor protein activity was determined by the standard assay as described under Methods in the presence and absence of 1 μM $CaCl_2$ to determine the units of PMI and CaBI. [b] Estimated from densitometer tracings of stained polyacrylamide gels as shown in Figure 1; in this case, the units (arbitrary) were from the tracings. [c] Not determined by the ATPase assay method; densitometer tracings showed amounts of PMI between those of the ETP_H particles and the A particles (Figure 1).

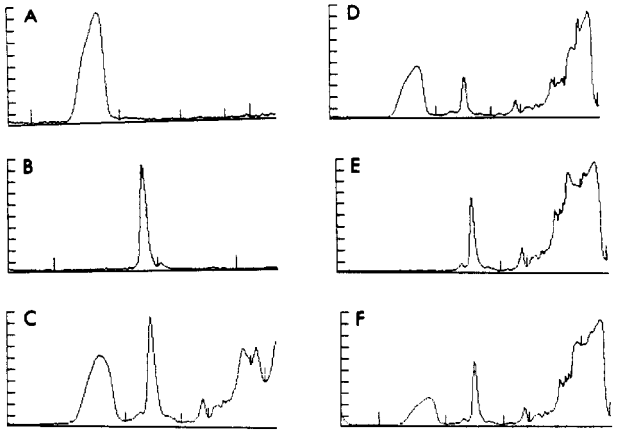

FIGURE 1: Densitometer tracings of stained polyacrylamide gels to determine the relative amounts of CaBI and PMI. Heat-stable protein fractions were prepared from submitochondrial particles (Yamada & Huzel, 1988). After electrophoresis (Laemmli, 1970), the gels were stained for 0.5 h with 0.1% Coomassie blue R-250 in 40% methanol and 10% acetic acid. The gels were destained with 40% methanol and 10% acetic acid for 1–3 h. Panel A: 2 μg of purified CaBI. Panel B: 2 μg of purified PMI. Panels C–F contained, in order, 10 μg of the heat-stable protein fraction from A particles, CaBI-supplemented A, CaBI-depleted A particles, and ETP_H particles. The top of the gel is at the right of each panel. An integrator gave the areas under each peak.

medium to inactivate CaBI and give an estimate of PMI activity only. Total inhibitor units were determined in the absence of exogenous Ca^{2+} (Yamada & Huzel, 1988).

It is apparent from Table I that the CaBI:PMI ratios as estimated by the two methods are in good agreement. This is the first report that ETP_H particles contain CaBI as well as PMI. Less heat-stable protein fraction was recovered from the ETP_H particles than from the A particles; there was a slight excess of CaBI over PMI in both. As expected, the amount of CaBI in the fraction from the CaBI-supplemented A particles was in large excess over PMI as well as over that of the other particles.

ATP Synthase and ATPase Activities of Submitochondrial Particles. It is apparent from Table II that ATP synthase activities of the intact particles were higher than those reported for another type of submitochondrial particle with succinate as respiratory substrate (Matsuno-Yagi & Hatefi, 1985). The results for ETP_H and A particles indicate that small increases in ATPase activity are not necessarily reflected in decreased ATP synthase activity. The synthetic activity was higher for ETP_H particles than for A particles. ETP_H particles are known to contain higher concentrations of certain components of the electron-transport chain such as NADH dehydrogenase. However, A particles are not lacking in the ATP synthase·ATPase complex (Yamada et al., 1981). Despite whatever is believed to be lacking from the A particles, it was found that addition of CaBI enhanced ATP synthase activity by 1.7-fold

Table II: Comparison of the ATP Synthase and ATPase Activities of Submitochondrial Particles of Bovine Heart[a]

fraction	ATP synthase act. (nmol min^{-1} mg^{-1})	ATPase act. (μmol min^{-1} mg^{-1})	
		+rotenone	+antimycin
A particles	697.0	3.27	3.29
ETP_H particles	944.5	4.56	4.11
CaBI-supplemented A particles	1194.0	0.33	0.29
CaBI-depleted A particles[b]	286.0	27.19	16.11
+0.2 μg of CaBI	502.7		2.66
+0.4 μg of CaBI	615.5		1.70
+0.2 μg of CaBI + 1 μM Ca^{2+}	280.0		15.90

[a] Enzyme activities were determined by the luminescence methods described under Methods. When rotenone was omitted from the standard reaction mixture, it was replaced by antimycin (1.67 μg/mL). [b] CaBI-depleted A particles (283 μg) were reconstituted with CaBI in 0.5 mM Mg^{2+}–ATP, 0.25 M sucrose, and 15 mM Tris/TES buffer (pH 6.5) in a final volume of 0.5 mL. Incubation was for 20 min at 25 °C. Aliquots were then removed for the standard assays of ATP synthase and ATPase activities by luminescence.

over that of the A particles to levels comparable to those found in the ETP_H particles. There was a concomitant reduction in ATPase activity of over 90% to much lower levels than those of the ETP_H particles. On the other hand, removal of CaBI resulted in a very large increase in ATPase activity concomitantly with a significant decrease in ATP synthase activity. In this case, ATPase activity with rottenone present was almost twice that found when antimycin replaced rotenone. Perhaps the protonmotive force generated by the high ATPase activity allowed proton reentry into the matrix with rotenone present to accelerate the ATPase activity further [see Nicholls (1982)].

CaBI restored the ATP synthase activity of CaBI-depleted A particles, and there was a concomitant suppression of ATPase activity to levels comparable to those of the original A particles. Restoration of the activities was negligible, however, in the presence of 1 μM Ca^{2+} (Table II). This critical concentration of Ca^{2+} caused formation of the inactive monomeric form of CaBI and also prevented binding to the ATP synthase·ATPase complex (Yamada et al., 1980, 1984; Yamada & Huzel, 1988).

Effect of Ca^{2+} Levels on ATP Synthase Activity of Submitochondrial Particles. Figure 2 shows that Ca^{2+} decreased profoundly the ATP synthase activity of the particles that contained CaBI but had no significant effect on the CaBI-depleted A particles. Maximal decrease in activity occurred with 1 μM Ca^{2+} at which all activities converged to a point close to the ATP synthase activity of the CaBI-depleted A particles. The inhibition by Ca^{2+} was reversible; as Ca^{2+} concentration was increased beyond 1 μM, ATP synthase activity gradually increased until the original was again achieved (not shown).

Figure 2 (inset) describes the time relationship of the ATP synthase activity of ETP_H particles. Activity was a linear function of time with or without Ca^{2+}. There was no sug-

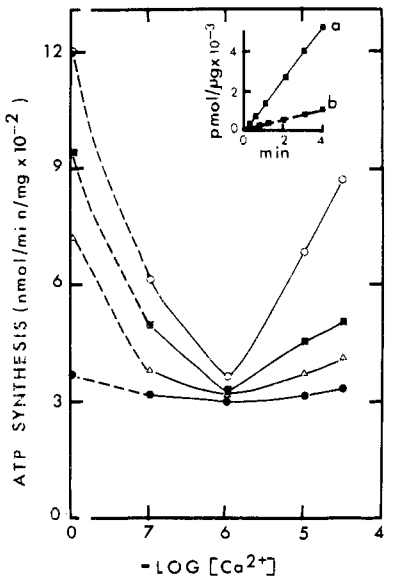

FIGURE 2: ATP synthase activity of submitochondrial particles as a function of Ca^{2+} concentration. The standard luminescence assay (Methods) was used. Ca^{2+}, as specified, was added to the preincubation medium. Controls were preincubated in the absence of Ca^{2+}. CaBI-supplemented A particles (○); ETP_H particles (■); A particles (△); CaBI-depleted A particles (●). Inset: Time relationship of ATP synthase activity of ETP_H particles in the absence of Ca^{2+} (a) and in the presence of 10^{-6} M Ca^{2+} (b).

Table III: Effect of Ca^{2+} on ATPase Activities of Submitochondrial Particles of Bovine Heart[a]

	ATPase act. (μmol min^{-1} mg^{-1})			
$[Ca^{2+}]$ (M)	ETP_H particles	A particles	CaBI-supplemented A	CaBI-depleted A
0	4.56	3.27	0.33	27.19
10^{-7}	11.18	11.14	8.43	27.80
10^{-6}	31.22	24.64	22.88	28.90
5×10^{-5}	23.24	17.07	11.85	27.67
10^{-4}	7.34	5.02	0.50	27.30
10^{-6} M + 1 mM EGTA	4.60	3.31	0.33	27.20

[a] The standard luminescence assay (Methods) was used with rotenone in the reaction mixture and Mg^{2+} at optimal concentrations. Ca^{2+}, as specified, was added to the preincubation medium. Controls were preincubated in the absence of Ca^{2+}.

gestion of cycling of product ATP by buildup followed by degradation.

Effect of Ca^{2+} Concentration on ATPase Activity of Submitochondrial Particles. Table III shows that the effect of $[Ca^{2+}]$ on ATPase activity was the reverse of that found for ATP synthase activity but only for those particles that contained CaBI. Again, the greatest effect was at 1 μM Ca^{2+} but in the reverse direction to ATP synthase. Ca^{2+} stimulation of ATPase activity was reversed progressively as the concentration of Ca^{2+} was increased over 1 μM. That the increases were due to Ca^{2+} was shown by the negation of the effect by EGTA. There was a negligible effect of Ca^{2+} on ATPase activity of CaBI-depleted A particles.

Discussion

The data are in agreement with the view that CaBI is required in addition to PMI to maintain the rates of ATP synthesis by submitochondrial particles of bovine heart. With every change in ATP synthetic activity, ATP hydrolytic activity responded in a reciprocal manner. It is worth noting that similar reaction mixtures and procedures were used to measure the forward and reverse reactions in the present work. This was not the case in some studies of PMI in which it was reported that ATP synthesis and hydrolysis rose together upon release of PMI (Lippe et al., 1988a).

That the effect of Ca^{2+} on depression of ATP synthesis and increase in ATP hydrolysis was through the inactivation of CaBI was supported by the finding that Ca^{2+} had no significant effect on the synthetic or hydrolytic activities of CaBI-depleted A particles. Ca^{2+} modulation of the activity of CaBI may well explain the Ca^{2+}-mediated diversion of substrate oxidation from ATP synthesis to Ca^{2+} uptake with a concomitant stimulation of ATP hydrolysis by mitochondria (Rossi & Lehninger, 1964; Vercesi et al., 1978; Hunter et al., 1976). CaBI would be gradually inactivated as mitochondrial Ca^{2+} concentration is raised from resting levels (Carafoli, 1987; Becker, 1981) to the micromolar range, at which concentrations certain dehydrogenases of the citric acid cycle are activated (Hanford, 1985). ATP synthesis would be diverted to hydrolysis which would provide energy to maintain Ca^{2+} levels (Lehninger et al., 1978; Tedeschi, 1981; Carafoli et al., 1980). CaBI would again become activated at higher $[Ca^{2+}]$ to suppress ATP hydrolysis and stimulate ATP synthesis. In this regard, CaBI is required in addition to PMI in order to respond to Ca^{2+} which PMI is unable to do (Klein et al., 1982).

Another role of CaBI may be to be in reserve to suppress ATP hydrolysis when PMI is translocated to a new site at the beginning of ATP synthesis (Klein & Vignais, 1983; Gomez-Puyou, 1981) or is released (Lippe et al., 1988a,b).

References

Becker, G. L. (1981) in *Calcium and Phosphate Transport Across Biomembranes* (Bronner, F., & Peterlik, M., Eds.) pp 79–82, Academic Press, New York.

Bygrave, F. L. (1977) *Curr. Top. Bioenerg. 6*, 259–318.

Carafoli, E. (1987) *Annu. Rev. Biochem. 56*, 395–433.

Carafoli, E., Gavilanes, M., Affolter, H., Tuena de Gomez-Puyou, M., & Gomez-Puyou, A. (1980) *Cell Calcium 1*, 255–265.

Fessenden, J. M., & Racker, E. (1966) *J. Biol. Chem. 241*, 2483–2489.

Frangione, B., Rosenwasser, E., Penefsky, H. S., & Pullman, M. E. (1981) *Proc. Natl. Acad. Sci. U.S.A. 78*, 7403–7407.

Gomez-Puyou, M. (1981) *Biochem. Biophys. Res. Commun. 100*, 400–406.

Green, D. E., & Ziegler, D. M. (1967) *Methods Enzymol. 10*, 416–424.

Hanford, R. G. (1985) *Rev. Physiol. Biochem. Pharmacol. 102*, 1–72.

Harris, D. A., Von Tscharner, V., & Radda, G. K. (1979) *Biochim. Biophys. Acta 548*, 72–84.

Horstman, L. L., & Racker, E. (1970) *J. Biol. Chem. 245*, 1336–1344.

Hunter, D. R., Haworth, R. A., & Southard, J. H. (1976) *J. Biol. Chem. 251*, 5069–5077.

Klein, G., & Vignais, P. V. (1983) *J. Bioenerg. Biomembr. 15*, 347–362.

Klein, G., Satre, M., Zaccai, G., & Vignais, P. V. (1982) *Biochim. Biophys. Acta 681*, 226–232.

Knowles, A. F., & Penefsky, H. S. (1972) *J. Biol. Chem. 247*, 6624–6630.

Laemmli, U. K. (1970) *Nature 227*, 680–685.

Lehninger, A. L., Reynafarje, B., Vercesi, A., & Tew, W. P. (1978) *Ann. N.Y. Acad. Sci. 307*, 160–176.

Lemasters, J. J., & Hackenbrock, C. R. (1976) *Eur. J. Biochem. 67*, 1–10.

Lienhard, G. E., & Secemski, I. I. (1973) *J. Biol. Chem. 248*, 1121–1123.

Lippe, G., Sorgato, M. C., & Harris, D. A. (1988a) *Biochim. Biophys. Acta 933*, 1-11.
Lippe, G., Sorgato, M. C., & Harris, D. A. (1988b) *Biochim. Biophys. Acta 933*, 12-31.
Matsuno-Yagi, A., & Hatefi, Y. (1985) *J. Biol. Chem. 260*, 14424-14427.
Nicholls, D. G. (1982) *Bioenergetics*, Academic Press, New York.
Panchenko, M. V., & Vinogradov, A. D. (1985) *FEBS Lett. 184*, 226-230.
Penin, F., Di Pietro, A., Godinot, C., & Gautheron, D. C. (1988) *Biochemistry 27*, 8969-8974.
Pullman, M. E., & Monroy, G. C. (1963) *J. Biol. Chem. 238*, 3762-3769.
Rossi, C. S., & Lehninger, A. L. (1964) *J. Biol. Chem. 239*, 3971-3980.
Schwerzmann, K., & Pedersen, P. L. (1986) *Arch. Biochem. Biophys. 250*, 1-18.
Tedeschi, H. (1981) *Biochim. Biophys. Acta 639*, 157-196.
Vercesi, A., Reynafarje, B., & Lehninger, A. L. (1978) *J. Biol. Chem. 253*, 6379-6385.
Yamada, E. W., & Huzel, N. J. (1988) *J. Biol. Chem. 263*, 11498-11503.
Yamada, E. W., Shiffman, F. H., & Huzel, N. J. (1980) *J. Biol. Chem. 255*, 267-273.
Yamada, E. W., Huzel, N. J., & Dickison, J. C. (1981) *J. Biol. Chem. 256*, 10203-10207.

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†

Åke Strid* and Pål Nyrén
Institutionen för Biokemi, Arrheniuslaboratorierna för Naturvetenskap, Stockholms Universitet, S-106 91 Stockholm, Sweden
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.

0006-2960/89/0428-9718$01.50/0